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**Life History Characteristics and Size Variation
of the Burrowing Mayfly Hexagenia (Ephemeroptera: Ephemeridae):
Maternal vs. Environmental Constraints**

by

Elizabeth Christene Hanes

A Thesis
submitted to the
Faculty of Graduate Studies and Research
through the Department of
Biological Sciences in Partial Fulfillment
of the requirements for the Degree
of Master of Science at the
University of Windsor

Windsor, Ontario, Canada

1992



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ABSTRACT

There is tremendous size variation within both laboratory and field populations of the mayfly Hexagenia. Because body size is so variable in Hexagenia, and because all life stages of this mayfly are both identifiable, and measurable, this insect is an excellent model organism for life history studies. I studied the size variation in adults (imagoes) of this mayfly collected in the summers of 1989 and 1990 from Lake St. Clair, Southwestern Ontario (42°20'N; 82°57'W). Additionally, I examined the influence of endogenous factors (egg hatching, egg diapause, maternal size) on size and survivorship of larval Hexagenia.

Female imagoes collected early in the emergence period were significantly larger than females collected later in the summer. There was low incidence of parthenogenesis (<5%), egg diapause (<0.01%) and viability of eggs produced through interspecific mating (0%) in the laboratory. Egg size of H. limbata was negatively correlated with collection date. Although egg size did vary among female sizes, there was no clear relationship between egg size and maternal size.

Maternal size was negatively correlated with larval size, after 40 d growth. Maternal size and (maternal size²) were correlated with per cent larval survival. Maternal parents exhibiting mean body length produced larvae with the highest survivorship after 40 d growth. The length of incubation (days) required for egg hatch was negatively associated with

per cent larval survival (40 d) but was positively correlated with larval size (80 d).

Of sediments examined from six different waterbodies, Saginaw Bay sediment provided larvae with the best survivorship and the largest size, and when given a choice, larvae selected that sediment type. Subsequently, I developed a standard sediment (STND-3) that mimiced the particle size distribution and organic content of Saginaw Bay sediment. Additionally, I developed a protocol for rearing large numbers of mayflies in the laboratory. I have also determined that using photographs as a means to estimate larval size is a valuable alternative to measuring actual larvae.

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GENERAL INTRODUCTION

Body size is an ecologically important characteristic that recently has received considerable attention in ecology (Peters 1983). Most studies of body size have focused on vertebrates, where determination of adult size has a large genetic component (Sebens 1987). However, invertebrates may be more suitable than vertebrates for growth studies because invertebrate growth rates and final sizes are not as constrained. The genetic component of invertebrates is often less important than the environmental conditions of invertebrates, and size can be influenced as these conditions change (Sebens 1987). Additionally, invertebrates spend a much larger fraction of their lives growing than do higher vertebrates (Peters 1983).

Adult insects exhibit considerable variation in adult body size (O'Neil and Skinner 1990). Body size can have important ecological and evolutionary implications for these organisms. For adult males, size can contribute to the outcome of aggressive interactions, mating success, (McLachlan 1986) and dispersal distance (Corkum 1987). For adult females, body size may determine reproductive value. The evolutionary significance of variation in adult male size lies in the association between maternal size and fecundity (Smith and Fretwell 1974, Peters 1983, Parker and Begon 1988).

Smith and Fretwell (1974) developed a model to predict offspring size and number to maximize an individual's fitness.

Accordingly, a large female should have more resources to devote to reproduction, and as such, should have either more eggs or larger eggs than a small female. However, it is also possible that large females have both larger eggs and more of them (see Berrigan 1991). Recent work on insect clutch size has assumed that egg size is constant and independent of clutch size (Parker and Begon 1986).

Although there is evidence that maternal size can influence important characteristics in offspring in vertebrates, few researchers have examined the influence of maternal size on offspring growth in insects (Parker and Begon 1986). Recent research focusing on the influence of maternal size on offspring in insects has concentrated on the influence of maternal size on diapause (Mousseau and Dingle 1991). Studies that involve maternal size and the allocation of maternal resources to progeny, rarely examine how the allocation ultimately influences offspring fitness. Researchers generally assume that propagule size reflects offspring fitness, with large size indicating greater fitness (Parker and Begon 1986).

There is tremendous size variation within both laboratory (Hanes and Ciborowski 1992, Corkum and Hanes 1992, D.C. Bedard, Ontario Ministry of the Environment, personal communication, M.G. Henry, Minnesota Cooperative Fish and Wildlife Research Unit, St. Paul, MN, personal communication) and field (Fremling 1973, Flannagan 1979, Heise et al. 1987, Schloesser and Hiltunen 1984) Hexagenia larval populations.

Accordingly, this mayfly is a good study organism for examining factors that influence size variation and how this size variation subsequently influences offspring size and survivorship.

Hexagenia (Ephemeroptera: Ephemeridae) are burrowing mayflies that dominate the benthic fauna of many large, shallow waterbodies throughout North America (Edmunds et al. 1976). Larval size variation can, in part, be attributed to the presence of multiple cohorts (Heise et al. 1987), delayed hatching of eggs (Britt 1955), differential growth of males and females (Wright et al. 1982), and a wide variability in growth of individuals from the same egg mass (Hunt 1953). In mayflies, all growth occurs during the larval stage; adult mayflies do not feed. Therefore, variation in larval size should influence adult size variation.

Early researchers were interested in Hexagenia because the adults were considered as 'pests'. During mass emergences, putrid-smelling drifts of adults can collect under lights, interfering with pedestrian, vehicular and boat traffic (Hunt 1953, Fremling 1960, Carlander et al. 1967). Hexagenia was also studied because of their economic importance. Larvae of Hexagenia are important members of the aquatic food chain, comprising the major constituent of many fish diets wherever they co-occur (Neave 1932, Hunt 1953, Fremling 1960, Needham et al. 1935).

More recently, interest in Hexagenia, in all life stages, has stemmed from its use in biomonitoring studies (egg:

Freisen 1979, larva: Hiltunen and Schloesser 1983, Henry et al. 1986, Landrum and Poore 1988, Hare et al. 1989, Bedard 1990, Edsall et al. 1991, Ciborowski et al. 1991, and adult: Clements and Kawatski 1984, Ciborowski and Corkum 1988, Kovats and Ciborowski 1989). Because of Hexagenia's close association with sediment, it is possible to measure contaminants in field-collected individuals to determine field levels of contaminants.

The length of the life cycle of this mayfly is variable and lasts from one to four years (Hunt 1953, Heise et al. 1987, Giberson and Rosenberg 1992a). Also, the number of larval instars is variable; 12-24 instars are recorded (Merritt and Cummins 1984). When mature, larvae swim to the water surface for the final larval moult, where the winged subimago emerges. Within 24 hours, the subimago moults to become the sexually mature imago (Hunt 1953). At dusk males form mating swarms. Mating is brief; females fly through the swarm, mate, oviposit eggs in the water, and die. Males are capable of mating several times, and although they usually are dead by morning, they may live for several days (Fremling 1960).

At present, although protocols for rearing Hexagenia larvae in the laboratory are available (Fremling 1967, Freisen 1981) very little standardization of the materials required for rearing exists. Of the materials required for rearing larvae (eggs and/or larvae, aquaria, water, sediment, air source, food) sediment type and source of larvae are probably

the most variable factors among laboratories.

A standard sediment, if widely used, could facilitate comparison of results among laboratories. A standardized method for laboratory-rearing larvae in large numbers also needs to be developed. For universal application of these standardized rearing procedures, the rearing methods should be practical alternatives to collecting sediment and larvae from the field.

This study was initiated to determine the influence of endogenous factors (such as egg hatching, egg diapause, and maternal size) on size and survivorship of larval Hexagenia, under controlled, laboratory conditions. In Chapter 1, I describe the results of this research. In chapter 2, I address the need for standardized materials (i.e., sediment, food, larvae) required for laboratory rearing of this mayfly.

1. LIFE HISTORY CHARACTERISTICS AND SIZE VARIATION
OF HEXAGENIA

INTRODUCTION

There is great size variation within both laboratory (Hanes and Ciborowski 1992, Corkum and Hanes 1992, D.C. Bedard, personal communication, M.G. Henry, personal communication) and field (Fremling 1973, Flannagan 1979, Heise et al. 1987, Schloesser and Hiltunen 1984) larval Hexagenia populations. In mayflies, all growth occurs during the larval stage; adult mayflies do not feed. Therefore, variation in larval size should influence adult size variation. Significant size variation in adult mayflies may be found over the course of the emergence season (Clifford and Boerger 1974) or at any one sample date.

Temperature is often cited as the most important factor influencing mayfly growth (for review, see Sweeney 1984). Recently, researchers have examined the influence of various exogenous factors (density, temperature, photoperiod, food quantity) on growth of Hexagenia larvae (Wright et al. 1982, McCafferty and Pereira 1984, Corkum and Hanes 1992, Giberson and Rosenberg 1992a, Hanes and Ciborowski 1992). Although all of these factors influence larval growth, exogenous factors are often only significant later in development (i.e., after 90 d growth or more, see Hanes and Ciborowski 1992).

Although less frequently studied, endogenous factors (day of egg hatching, maternal size, egg diapause, parthenogenic vs. fertilized eggs) also can influence larval growth. Hanes and Ciborowski (1992) found that day of egg hatching

influenced growth of Hexagenia larvae significantly; its influence acted early in larval development (at 30 and 60 d growth). They speculated that the most likely factors contributing to growth differences observed between larvae that required 6 vs. 7 d to hatch were either differences in maternal parent or inherent variation among larvae within single broods.

Maternal size may influence offspring growth since females of different sizes have different amounts of resources to allocate to offspring. Smith and Fretwell (1974) developed a model to predict the optimal balance between offspring size and number that maximizes an individual's fitness. Accordingly, large females should have more resources to devote to reproduction, and as such, should have either more eggs or larger eggs than a small female. However, it is also possible that large females have both more and larger eggs (see Berrigan 1991). A positive correlation between fecundity and maternal size has been observed for mayflies (Clifford and Boerger 1974). Larger females produce a greater number of eggs. However it is not known how egg size varies with maternal size. Although recent work with insects has focused on clutch size, researchers assume that egg size is constant and independent of clutch size (Parker and Begon 1986).

Although there is evidence that maternal size can influence important characteristics, such as growth and survival, in offspring in vertebrates, few researchers have examined the influence of maternal size on offspring growth

in insects (Parker and Begon 1986). It is generally assumed that larger egg size reflects a greater fitness of the offspring (Smith and Fretwell 1974). Rearing larvae hatched from eggs of maternal parents of different sizes would be required to determine the true relationship between maternal size and offspring growth.

Egg diapause can contribute to larval size variation by causing eggs to hatch at different times of the year (Rosenberg and Giberson 1992b, Mousseau and Dingle 1991). Giberson and Rosenberg (1992b) detected an egg diapause in a northern population (South Indian Lake, Manitoba) of H. limbata. In the laboratory, cold storage was required to stimulate hatching for almost one third of eggs studied. In some insects, the environmental conditions experienced by the maternal parent can stimulate egg diapause. For example, short photoperiods and cold temperatures experienced by the maternal parent can result in diapausing offspring, while long photoperiods and high temperatures can avert diapause in offspring (for review, see Mousseau and Dingle 1991).

This study was initiated to determine the amount of size variation present in adult Hexagenia collected from Lake St. Clair, Ontario. I also wanted to determine whether factors such as interspecific mating, parthenogenesis, and egg diapause exist; the presence of these factors could possibly contribute to size variation. Additionally, I wished to determine how endogenous factors such as maternal size and day of egg hatch contribute to larval, and ultimately adult size

variation and growth of offspring. Specifically, my objectives were to include:

- 1) Collecting and measuring adult Hexagenia from Lake St. Clair to ascertain size variation in adults;
- 2) Monitoring egg hatch from adults collected at Lake St. Clair; determining if there is a diapause for eggs in this population;
- 3) Artificially inseminating female subimagos to determine if viable offspring production is possible between Hexagenia limbata and H. rigida;
- 4) Examining the influence of maternal size on:
 - a) egg size
 - b) first instar size
 - c) offspring growth
- 5) Examining the influence of day of egg hatch on larval growth.

METHODS AND RESULTS

I. Collection of Adult Mayflies and Eggs

Methods

A. Collection 1989-90

To obtain large numbers of eggs for experiments, adult imagoes were collected in the summer of 1989 from females emerging from Lake St. Clair, southwestern Ontario (42°20'N; 82°57'W). In order to avoid the effect of confounding factors such as date of emergence on egg development, collection was restricted to two dates during the peak adult emergence period (4 and 6 July 1989). Adult imagoes were collected using portable ultraviolet light traps (Kovats and Ciborowski 1989). The ultraviolet light attracts adult mayflies, which then land on a white sheet surrounding the light. Eggs are obtained from adults by placing gravid females into plastic bags partly-filled with aerated distilled water. When placed on the water surface, most female imagoes immediately release their eggs. A small amount of clay was added to prevent the eggs from clumping together, which can limit the amount of oxygen available to the eggs (M.G. Henry, Minnesota Cooperative Fish and Wildlife Research Unit, St. Paul, MN, personal communication). Once the female imagoes had released their eggs, they were removed and preserved in 70% ethanol (EtOH).

Eggs from groups of 20 or more females were amalgamated into 2-L storage bags containing aerated distilled water (=batch females' eggs). Additionally, eggs were collected

from individual females. The eggs obtained from the individual female collections were placed into separate 180 mL Whirl-pack[®] bags with aerated water. No clay was added to the water in these bags so that eggs could later be measured. When clay is added to mayfly eggs, the clay sticks to the eggs making accurate measurement impossible. All eggs were returned to the laboratory in plastic bags where they were cooled to 8°C in 4°C decrements (Friesen 1981).

Seventy individual female imagoes were collected on the 4 and 6 July 1989. Seventeen batch bags containing eggs from at least 20 females were also collected. These eggs provided larvae for all experiments conducted in 1989.

Adult male imagoes and subimagoes also were collected and preserved in 70% EtOH. Body length of males and females collected on each date was measured to the nearest 0.1 mm with vernier calipers. Females could be identified to the generic level only (W.P. McCafferty, Purdue University, W. Lafayette, IN, personal communication). The species identity of males was ascertained by examining the genitalia.

In summer 1990, a more extensive egg collection was undertaken from females emerging from Lake St. Clair (42°20'N; 82°57'W) (Table 1.1). Adult imagoes and subimagoes were collected on 7 occasions between late June and early August. Approximately 3 million eggs were obtained from a total of ca. 750 female imagoes. Mayflies were collected from a lighthouse located on the Canadian side of the Detroit River near the outflow of Lake St. Clair. The lighthouse attracted

Table 1.1. Summary of numbers (n) and mean size (mm) of the 1990 individual female imago collections and total number of male imagoes collected. The number in parenthesis indicates the standard error (S.E.). WK. indicates the week during the emergence period that the mayflies were collected.

| DATE | WK | INDIVID. FEMALE | | MALE IMAGOE | |
|---------|----|-----------------|---------|------------------|-------------------|
| | | | IMAGOE | <i>H. rigida</i> | <i>H. limbata</i> |
| 29 June | 1 | \bar{X} | 18.95 | 17.35 | 18.40 |
| | | S.E. | (0.308) | (0.179) | (0.234) |
| | | n | 49 | 69 | 33 |
| 7 July | 2 | \bar{X} | 18.81 | 18.78 | 19.71 |
| | | S.E. | (0.570) | (0.200) | (0.2710) |
| | | n | 45 | 33 | 22 |
| 18 July | 3 | \bar{X} | ---- | 17.55 | 18.40 |
| | | S.E. | | (0.749) | (0.221) |
| | | n | | 6 | 29 |
| 21 July | 4 | \bar{X} | *19.49 | ---- | 19.27 |
| | | S.E. | (0.290) | | (0.343) |
| | | n | 50 | | 21 |
| 23 July | 4 | \bar{X} | 16.89 | 17.46 | 18.66 |
| | | S.E. | (0.244) | (0.189) | (0.400) |
| | | n | 48 | 54 | 22 |
| 28 July | 5 | \bar{X} | 18.15 | ---- | 17.11 |
| | | S.E. | (0.314) | | (2.057) |
| | | n | 48 | | 9 |
| 5 Aug | 6 | \bar{X} | 21.55 | ---- | 18.57 |
| | | S.E. | (0.543) | | (0.680) |
| | | n | 11 | | 12 |

* no individual female imagoes were collected on this date. The mean size is determined from 50 females randomly selected from batch-collected females.

significantly more adults than the light trap did the previous year. At each collection date, whenever numbers permitted, eggs from 50 individual female imagoes were collected. In addition, batch bags containing eggs from at least 20 female imagoes were obtained. Again, male adults were collected and preserved in 70% EtOH. All adult mayflies were measured to the nearest 0.1 mm using vernier calipers. Two-way analysis of variance was used to determine if size of male imagoes differed between the species and among collection dates. One-way analysis of variance was conducted on female imagoes to determine if female size varied among collection dates.

B. Egg Hatching and Diapause

Eggs collected in July 1989 were used to determine whether differential hatching of eggs contributes to size variation observed in larval populations. To determine the possible influence of overwintering of eggs on delayed recruitment into natural populations, the effect of cold storage on egg hatch was examined.

At the time that eggs were returned to the laboratory after collection in 1989, approximately 100 eggs were removed from each of 100 bags containing single-female eggs and 30 bags containing batch eggs (Fig. 1.1). Half of the eggs ($n=50$) of each female were placed into individual petri dishes at room temperature while the other half were placed in other dishes and cooled to 8°C in 4°C decrements (Freisen 1981). The eggs kept at room temperature were monitored daily for hatch. As larvae hatched, they were removed from the petri

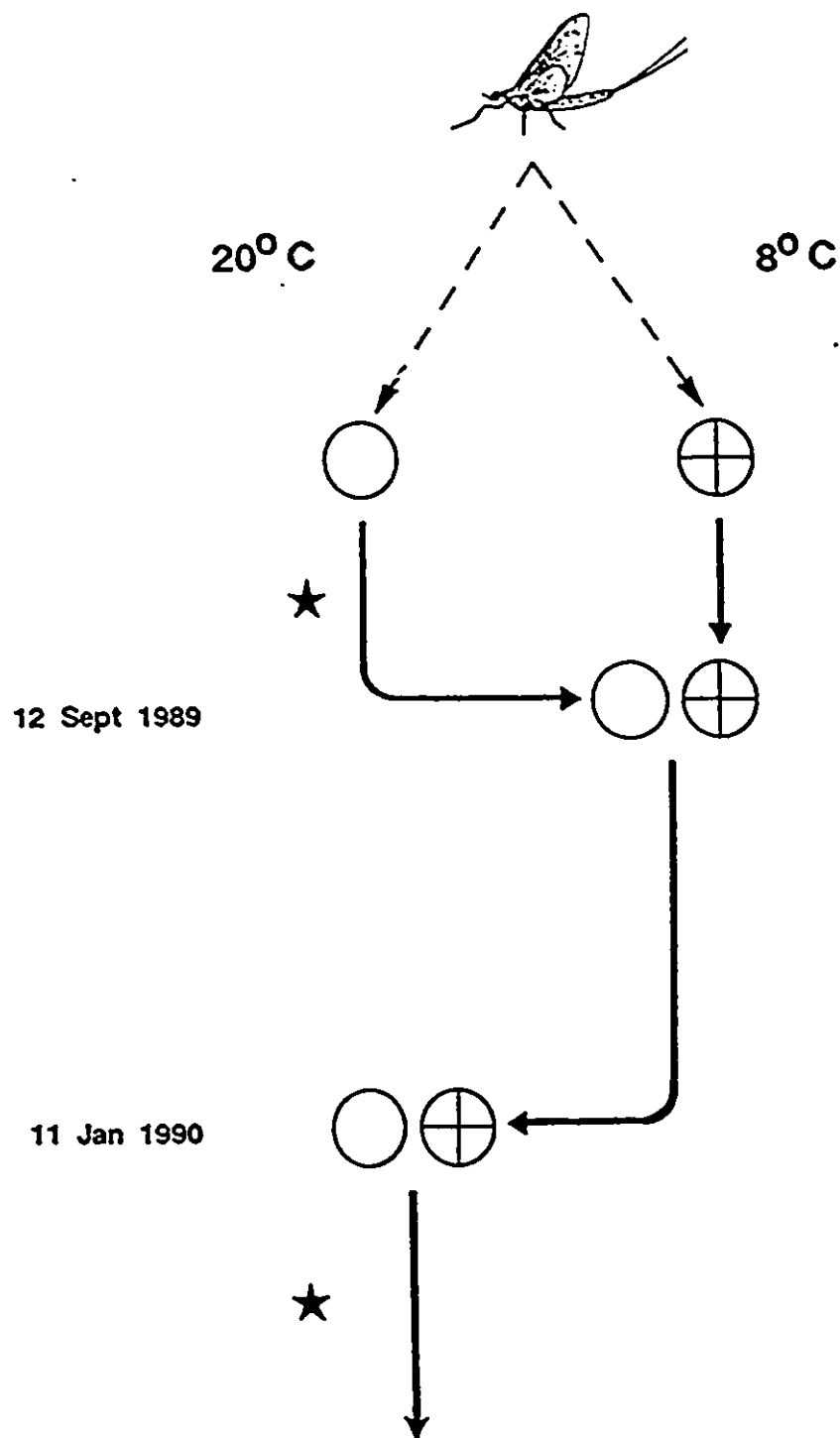


Fig. 1.1. Schematic diagram of procedure used to examine egg hatch at room temperature (20°C) and effect of cold storage (8°C). The stars indicate when hatch was monitored. Dates indicate when eggs were transferred from one temperature to another.

dish and enumerated. All unhatched eggs were left at room temperature for two months (D. Giberson, University of Prince Edward Island, personal communication); during this time very few additional eggs hatched. On 12 Sept 1989, all unhatched eggs were placed into cold storage (8°C). On 11 January 1990, these eggs, in addition to the eggs originally put in cold storage, were warmed to room temperature and monitored daily for hatching. Hatched larvae were recorded, enumerated and removed from each petri dish before being discarded.

C. Artificial Insemination

In 1990 (16 July - 23 July), male and female subimagos were hand-collected (at the same site on Lake St. Clair as the egg collection) and returned to the laboratory in plastic bags. These subimagos were maintained en masse within large nylon-mesh cages overnight, during which time many transformed to imagoes. In the morning, artificial insemination was conducted following procedures outlined by Friesen (1981). Eggs were obtained from female imagoes by severing the abdomen in front of the egg pockets (where the abdomen joins the thorax). Eggs were gently squeezed out of the abdomen into a petri dish containing a small amount (two drops) of Yeager's solution (Friesen 1981). Yeager's solution is a saline solution consisting of NaCl (10.93 g), KCl (1.57 g), CaCl₂ (0.83 g) and MgCl₂ (0.17 g) dissolved into 1 L distilled water (Yeager 1939). Eggs from one female were divided in three portions. One third was left unfertilized so that the

relative frequency of parthenogenic eggs could be estimated, one third was mixed with H. limbata sperm, and one third was mixed with H. rigida sperm. Sperm were collected from male subimagos or imagoes, by carefully dissecting out male genitalia into 1 drop Yaeger's solution. Sperm were then mixed with a portion of a female's eggs. The sperm and eggs remained in the Yaeger's solution for 10 min before aerated, distilled water was added to the now artificially inseminated eggs. A total of 41 attempts at artificially inseminating females' eggs were conducted (Table 1.2).

Each group of eggs was maintained in dechlorinated, deionized water at room temperature and monitored daily until no more hatching was observed. Hatching success was expressed as approximate proportion of eggs showing eclosion.

In addition to permitting species identification of the females and eggs, the possibility of interspecific mating could be detected by this design. Because H. rigida males were not captured on all dates, some females' eggs were inseminated with H. limbata sperm only (Table 1.2).

Table 1.2 Summary of 1990 artificial insemination experiments. Eggs from a female were split into 3 portions. One portion was unfertilized, a second portion was mixed with sperm from an H. limbata male, a third portion was mixed with sperm from an H. rigida male, if available.

| <u>Date</u> | <u>Males</u> | |
|-------------|-------------------|------------------|
| | <u>H. limbata</u> | <u>H. rigida</u> |
| 16 July | 6 | 6 |
| 18 July | 7 | 7 |
| 19 July | 2 | 0 |
| 21 July | 10 | 0 |
| 23 July | 16 | 10 |

Results

A. Mayfly Collection

(i) Summer 1989

The mean BL (± 1 S.E.) of female imagoes collected was 18.58 ± 0.331 mm. The size distribution of the female imagoes was weakly bimodal (Fig. 1.2).

Male imagoes collected on 4 and 6 July 1989 consisted of H. limbata (Serville) and H. rigida McDunnough in a 2:1 ratio ($n=250$). Species level identification was possible only for 6 July 1989 collection, as male imagoes and subimagoes were only present on this collection date; adult female mayflies cannot be used for taxonomic identification (W.P. McCafferty, Purdue University, personal communication).

(ii) Summer 1990

The imagoes consisted of H. limbata (Serville) and H. rigida McDunnough in a 5:4 ratio (based on proportions of males collected concurrently with females; $n = 286$).

The size frequency distribution of females was unimodal when all specimens were combined over the entire summer (Fig. 1.3). Females collected early in the emergence period were significantly larger than females collected later in the summer (Fig. 1.3; 1-way ANOVA, $P < 0.001$), but female size was not linearly related to calendar date of emergence (linear regression, $R^2 = 0.04$, $P > 0.5$).

Results of a two-way ANOVA revealed that there was significant variation among size of male imagoes between

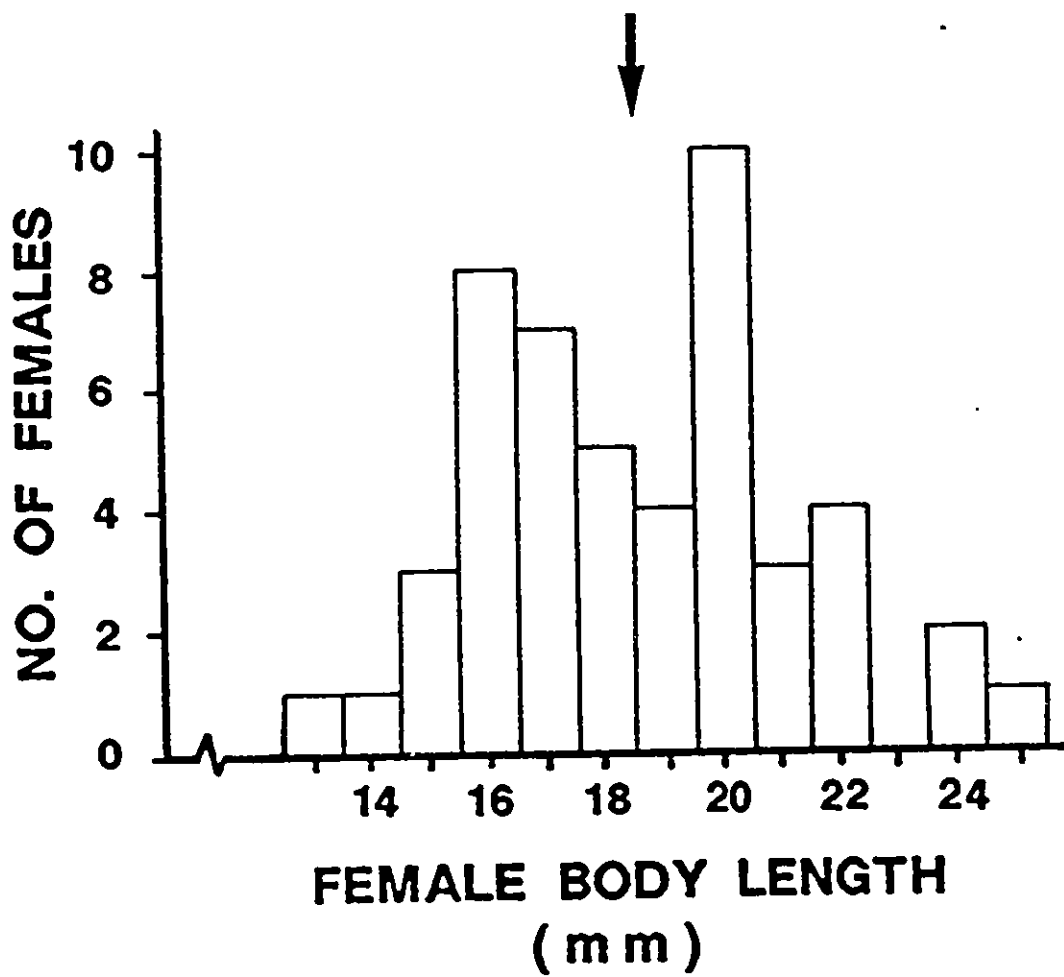


Fig. 1.2. Size distribution of *Hexagenia* female adults collected on 4 and 6 July 1989. The arrow indicates the mean female size (18.5 mm).

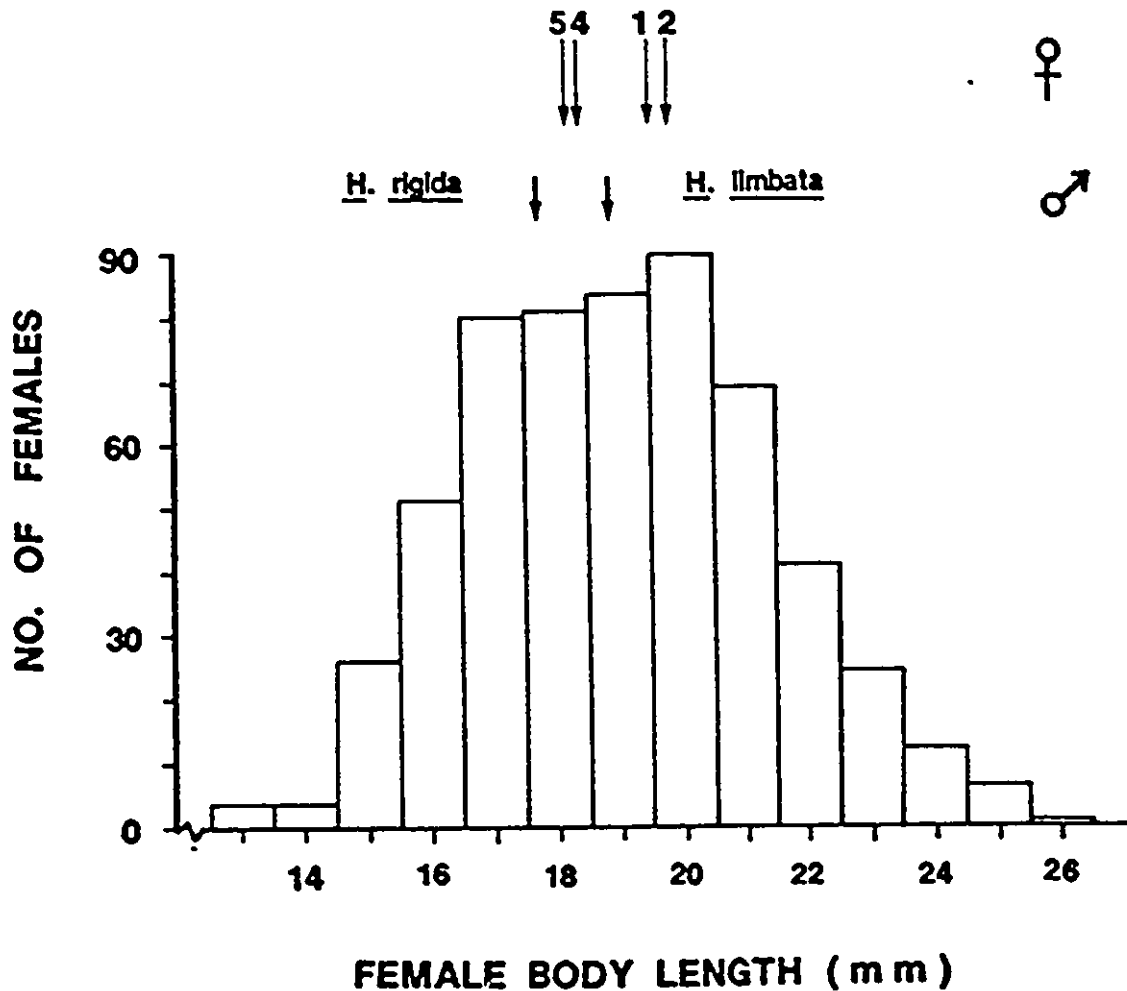


Fig. 1.3. Size frequency distribution of body length of females collected in 1990. Numerals over arrows indicating mean female length (mm) represent each of four dates, numbered chronologically (1= 29 June, 2= 7 July, 4= 21, 23 July, 5= 28 July. No female imagoes were collected during week 3). The lower pair of arrows indicate mean length of male H. rigida and H. limbata collected over entire summer.

species ($P < 0.001$) and among dates of emergence ($P < 0.001$, Table 1.3). Mean size of *H. rigida* male imagoes was approximately 1 mm smaller than mean size of *H. limbata* males on each collection date (Table 1.1). Although size of male imagoes did vary among date of emergence, male size was not linearly related to calendar date for either *H. limbata* or *H. rigida* (linear regression, $P > 0.05$). There was a significant interaction term between species and date of emergence (Two-way ANOVA, $P < 0.001$, Table 1.3).

B. Egg Hatching and Diapause

The eggs that were collected 4 and 6 July 1989, and kept at room temperature, hatched in two weeks. Approximately 90 - 95 % of all eggs hatched. Duration of hatching was approximately 4 d; after this time, very few additional larvae hatched.

After six months at 8°C, the percentage of cold-stored hatch for the eggs that subsequently hatched after warming was very high; 90-95% of the eggs successfully hatched. Eggs began to hatch five days after warmed to room temperature and hatching was complete within 4-5 days. Of the eggs that were originally left at room temperature to hatch, very few additional hatchings were observed ($< 0.01\%$). Thus, cold storage prevented immediate egg hatching, but had no influence (either positive or negative) on egg viability after six months.

Table 1.3. Summary of two-way ANOVA of the influence of species, date of emergence (Date), and their interaction on male imago size. Analysis conducted on male imagoes collected in June - July 1990.

| VARIABLE | df | S.S. | F | p |
|-------------|-----|---------|--------|---------|
| Species | 1 | 1334.3 | 21.010 | < 0.001 |
| Date | 3 | 5150.8 | 27.035 | < 0.001 |
| Interaction | 3 | 4540.7 | 23.833 | < 0.001 |
| Error | 462 | 33532.8 | | |

C. Artificial Insemination

A total of 41 crosses of Hexagenia spp. female imagoes with H. limbata and H. rigida male imagoes was performed. Eggs began to hatch 14 d after insemination, and hatching continued for approximately 14 d after this.

The attempts at artificial insemination met with only limited success. No hatching was observed in 7 of the 41 crosses (Table 1.4). In 7 trials, all eggs hatched in all three treatments (unfertilized, H. limbata sperm, H. rigida sperm). These females may have produced parthenogenetic eggs. However, the viability of eggs hatching in these trials (usually 100 %) was much higher than was observed in trials with other females (<5 %, Freisen 1981), suggesting that these 7 females had previously mated.

Eggs of 11 females had higher viability when mixed with one type of sperm than unfertilized eggs or eggs mixed with the other species' sperm (Table 1.5). Ten of these females were apparently H. limbata and only one was H. rigida (Table 1.5). This suggests that either H. limbata females were much more common in the collections or that eggs of H. rigida females did not respond well to the artificial insemination methods. The incidence of successful hatching was variable among females and among collection dates, ranging from <5 to 100 percent.

Apparently parthenogenetic eggs were observed for 11 of the 34 females not suspected of having mated previously (Table 1.5). Viability of these eggs was generally low

Table 1.4. Results of artificial insemination trials. 'No hatching', crosses in which no eggs hatched in any treatment; '100% Hatching', crosses in which all eggs hatched in all treatments; '< 100 % Hatching, Equiv. all Treat.', crosses in which the maximum proportions of eggs hatching were the same in two or more of the 3 treatments; 'Betw. Treat. Differences', crosses in which species identity could be determined. Dates represent calendar dates in 1990 on which adults were collected. Crosses involving no *H. rigida* males are listed in the lower half of the table.

| Date (July) | No. Crosses | No Hatching | 100% Hatching | <100% Hatching Equiv. all Treat. | Betw. Treat. Differences |
|----------------|----------------|----------------|------------------|-------------------------------------|-----------------------------|
| 16 | 6 | 2 | 3 | 0 | 1 |
| 18 | 7 | 2 | 1 | 1 | 3 |
| 23 | 10 | 1 | 0 | 7 | 2 |
| 19 | 2 | 0 | 0 | 2 | 0 |
| 21 | 10 | 0 | 1 | 5 | 4 |
| 23 | 6 | 2 | 2 | 1 | 1 |
| Total | 41 | 7 | 7 | 16 | 11 |

Table 1.5. Results of artificial insemination trials. Entries listed under a species name represent crosses in which more eggs hatched in that treatment than in the other treatment(s). 'Parthenogenetic' cases were those in which at least some (but <100%) eggs hatched in the unfertilized treatment. Remaining explanation as in Table 3A.

| Date (July) | Total No. Crosses | Female <i>H. limbata</i> Freq. of % of eggs Crosses Hatching | Female <i>H. rigida</i> Freq. of % of eggs Crosses Hatching | Parthenogenetic Freq. of % of eggs Crosses Hatching |
|----------------|-------------------------|--|---|---|
| 16 | 6 | 1 25 | 0 -- | 0/3 -- |
| 18 | 7 | 2 <5-25 | 1 <5% | 1/6 <5 |
| 23 | 10 | 2 100 | 0 -- | 2/10 <5-10 |
| 19 | 2 | 0 -- | N/A | 1/2 <5 |
| 21 | 10 | 4 50-100 | N/A | 7/9 <5-20 |
| 23 | 6 | 1 50-100 | N/A | 0/4 0 |
| Total | 41 | 10 | 1 | 11/34 |

(usually <5 percent), but two females' eggs exhibited up to 20 percent incidence of hatching without fertilization.

II. Standard Laboratory Rearing Methods

A. Rearing Containers

Studies of variation in larval development and survival were carried out in the laboratory. Typically, larvae were reared under standardized conditions during laboratory rearing experiments. Except for the mass-rearing experiment (Chapter 2, Part III), larvae were reared in either 500-mL styrofoam or 1-L plastic containers. When larvae are reared using a different method, the rearing technique is described in the pertinent section.

When 500-mL styrofoam containers (diameter, 10.8 cm; depth, 6.8 cm) were used, a one-cm depth of sediment was placed into each rearing tank (Friesen 1981). Unless otherwise indicated, the sediment used was a 2:1 dry w/w mixture of potting soil and potter's clay (= STND-1 sediment) (M.G. Henry, Minnesota Cooperative Fish and Wildlife Research Unit, St. Paul, MN, personal communication) (this chapter, part II D). I have assessed the suitability of this sediment type compared to field collected sediments and to other synthetic sediments (Chapter 2, Part I). When 1-L plastic containers (diameter, 10 cm; depth 13 cm) were used, a 2.5 cm depth of sediment was placed into each tank. A greater amount of sediment was required in the larger tanks since these tanks were used for long rearing periods (i.e. >120 d).

B. Sources of Larvae

Whenever larvae were required for experiments, eggs were removed from cold storage and placed into petri dishes containing aerated distilled water. Eggs were maintained at room temperature and monitored daily for hatching. Typically, on the second day of egg hatching, one- and two-day-old larvae were inoculated into rearing tanks (7 larvae/500 mL tank or 8 larvae/1 L tank). Rearing larvae at this density results in larvae that grow quickly and maintain high survivorship (Hanes and Ciborowski 1992).

C. Food Composition and Synthesis

Successful growth and development of laboratory-reared animals depends upon application of appropriate quantities of a suitable diet. M. Henry (Minnesota Cooperative Fish and Wildlife Research Unit, St. Paul, MN, unpublished data) has conducted extensive experiments on growth responses of Missouri Hexagenia and developed a successful food (liquid suspension of Tetramin[®], Alfalfa powder and yeast) and feeding regimen that maximizes laboratory growth. Hanes and Ciborowski (1992) reported excellent growth and survival of Hexagenia larvae reared on this diet. Thus, laboratory-reared larvae were fed this food mixture twice weekly as an aerosol. Food supply to each tank was kept constant by agitating the base of the food container with a magnetic stirrer.

D. Standard Sediment and Synthesis

M.G. Henry (Minnesota Cooperative Fish and Wildlife Research Unit, St. Paul, MN, personal communication). proposed using plant potting soil as a rearing medium for Hexagenia rigida larvae. Hanes and Ciborowski (1992) achieved excellent rearing success with potting soil and potter's clay. This standard sediment (STND-1) contains potting soil and potter's clay in a 2:1 ratio (dry wt).

To prepare the STND-1 sediment, the potting soil (Zehr's "No Name" Potting Soil^R) was air dried and then sieved through a No. 10 (1 mm) sieve. The mass of the sieved soil fraction was determined and corrected for per cent moisture. This mass is used to calculate the required amount of potter's clay, which was also corrected for water content. The potter's clay was then mixed with distilled water in a blender to the consistency of a thick slurry. This clay slurry was mixed with the sieved soil until a homogeneous mixture was obtained. The wet sediment was air dried until damp and then sieved through a No. 5 sieve (2 mm). This resulted in a coarse-powdered sediment that was easy to manipulate and which, when completely dried, could be stored covered, indefinitely. The sediment was autoclaved (20 min at 120°C, 103 kPa) before being used in experiments.

E. Maintenance

After sediment was added to the rearing tanks they were filled with aerated distilled water. Water was aerated for

at least 24 h, with filtered air from a laboratory air line or portable air pump, prior to use. As required, deionized water was added to rearing tanks to compensate for evaporation. After every 60 d, half of the water was removed from rearing tanks and replaced with fresh aerated water. Tanks were aerated continuously using a network of Tygon[®] tubing and fine capillary tubing connected to an air source (Corkum and Hanes 1989). The tanks were kept under a 16:8 L:D (light:dark) photoperiod beneath fluorescent lights. Water temperature was maintained between 16 and 22° C, which is within the optimal range for Hexagenia (Wright and Mattice 1981b). Values of dissolved oxygen, conductivity and pH were determined at regular intervals throughout all experiments.

F. Recovery

Larvae were retrieved from tanks by carefully pouring off overlying water through a 250 µm sieve and replacing it with carbonated water. Anaesthetized larvae rose to the water surface and could then be removed easily using a Pasteur pipette. If all larvae were not retrieved from a tank using this method, sieving the sediment through a 250 µm sieve was necessary. Sediment was removed from the sieve and placed into petri dishes in small, manageable quantities. Sediment was scanned, by eye, for larvae. Unless required alive, larvae were preserved in 70% ethanol.

Head width (HW) [measured across the eyes], and body length (BL) [total length excluding cerci] was determined for

all larvae using an ocular micrometer and a dissecting microscope to the nearest 0.075 mm. All measurements were made on preserved animals. In addition, sex was determined for larvae as soon as male genitalia could be distinguished (body length 6-7 mm). Relative survivorship (no. larvae recovered/no. initially added) was calculated for larvae within each tank.

G. Data Analysis

Unless otherwise stated, I used head width (HW) rather than body length as a measure of larval size. Head width of Hexagenia larvae is a more precise indicator of body size (lower coefficient of variation, (V) than total BL [V for HW = 23.184%; V for BL = 25.724%] Hanes and Ciborowski 1992). Additionally, since results of nested analysis of variance (ANOVA) usually indicated significant variation in HW among tanks within treatments compared to variation among larvae within tanks, unless otherwise stated the mean HW of all larvae within each tank was calculated and used as the size variate for analysis (Sokal and Rohlf 1981). Thus, sample sizes (n) typically refer to the number of replicate rearing tanks employed, not to the number of larvae per tank. Specific data analyses are described in detail in each section.

III. Influence of Maternal Size on Egg Size, Newly-hatched Larvae and Size/Survivorship of Offspring

Methods

A. Egg Size

(i). Pilot Study

To evaluate among-female variation in egg size, single-female bags of eggs were selected from the collections made throughout the 1990 emergence period. Eggs from 8 females belonging to each of 7 1-mm size classes (body length, 15-21 mm) were randomly selected without specific regard to emergence date. A subsample of eggs from each bag was removed with a Pasteur pipette and mounted on a microscope slide using CMC-9AF aqueous mounting medium (Master's Chemical Co., Des Plaines, IL). Uneven evaporation of medium from some slides caused air bubbles and distortion of the eggs. Accordingly, between 5 and 8 slides per size class were ultimately examined. Twenty eggs per slide (i.e., per female) were measured.

Species identity was determined by examining the pattern of chorionic sculpturing on the egg surfaces of each female. Chorionic reticulations of *H. limbata* are straight, whereas those of *H. rigida* are sinuous (Fig. 1.4; Neave 1932, Koss 1974). Length and width of each egg was measured to the nearest 5 μ m with an ocular micrometer at 100X magnification on a compound microscope. Egg size was expressed as visible area of the ellipse (product of π X one-half length X one-half width).

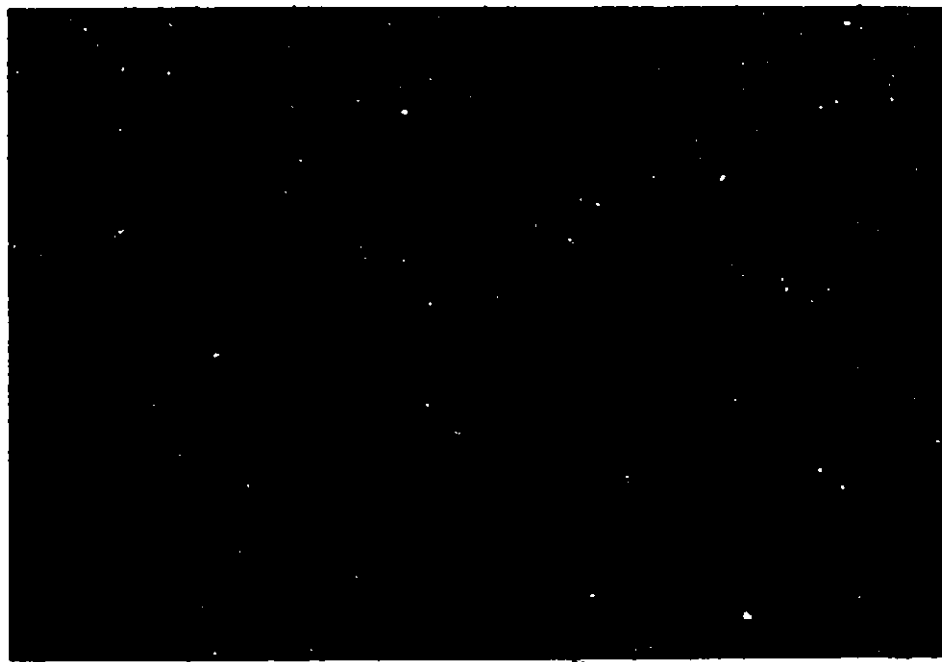


Fig. 1.4. Photographs of Hexagenia eggs stained with CMC-9AF mounting medium demonstrating a) H. limbata eggs with straight vs. b) H. rigida eggs with sinuous chorionic sculpturing.

Since the mayfly eggs were mounted on standard microscopic slides and not depression slides, the pressure of the coverslip may have inflated the length and width measurements of the eggs (Koss 1968). Therefore, the egg sizes indicated in this study may be slightly larger than actual. Indeed, the size range of eggs for my study overlaps, but is larger than the size range of Hexagenia eggs reported in the literature (my study: length range, 260-340 μm , width range: 175-260 μm ; Koss 1968, length range, 250-300 μm , width range, 150-200 μm). Since the degree of egg enlargement should be consistent among all replicates, analyses conducted on these measurements are appropriate.

Regression analysis was used to determine the extent to which female size, collection date and species influence egg size.

(ii) Main Study

To clarify the relationship between female size and egg size and date of emergence and egg size, a more extensive investigation was conducted on the eggs collected in 1990. A complete 7 (female size) x 4 (date of emergence) factorial design was used (Table 1.6). In January 1991, the eggs from 5 females for each female size class per 1990 collection date, were mounted on microscope slides. The 'visible area' of 30 eggs from each female was measured.

The data were analyzed by multiple regression similarly to the pilot study (this chapter, III A (i)).

Table 1.6. Experimental design examining the influence of female size and date of emergence on egg size. 5 females were examined per cell and 30 eggs per female were measured.

| DATE | FEMALE SIZE (mm) | | | | | | |
|---------|------------------|-------------|-------------|-------------|-------------|-------------|-------------|
| | 16 | 17 | 18 | 19 | 20 | 21 | 22 |
| 29 June | n=5 (30) | n=5 (30) | n=5 (30) | n=5 (30) | n=5 (30) | n=5 (30) | n=5 (30) |
| 7 July | " | " | " | " | " | " | " |
| 21 July | " | " | " | " | " | " | " |
| 28 July | " | " | " | " | " | " | " |

B. Newly-hatched Larvae

Forty females were selected from the individual collections of summer 1989 for the main maternal study (see below). The offspring of these females were used to examine the influence of maternal size on the size of newly-hatched larvae. Eggs from five females belonging to each of 8 1-mm size classes (body length, 15-22 mm) were removed from cold storage. On the second day after hatching had begun, larvae were added to the rearing tanks for the maternal study (see below: main maternal study). Any larvae that had hatched and were not required for experiments were removed and preserved in 70% ETOH. Five of these larvae from each female of each of the 8 1-mm size classes were measured to the nearest 0.075 mm using an ocular micrometer and a dissecting microscope (HW across the eye, BL excluding cerci).

C. Size/Survivorship of Offspring

(i) Pilot Study

a. Individual Females

Hanes and Ciborowski (1992) detected differences in survival and growth among larvae emerging from simultaneously incubated eggs that varied by as little as one day's hatching time. A pilot study was conducted to determine the effect of maternal parent, maternal size and day of hatch on the size distribution of Hexage 1a larvae (Table 1.7a).

Eggs (ca. 200) obtained from 12 individual female imagoes representing a range of body sizes (see below) were set out

Table 1.7. Experimental design for pilot studies examining effects of maternal parent, maternal parent size, and day of hatch on larval size and mortality.

a. Eggs obtained from individual female imagoes. Four female imagoes were 18.5 mm (mean (\bar{X}) length), three were 21 mm ($\bar{X} + 1$ S.E.), four were 16 mm ($\bar{X} - 1$ S.E.).

| Hatch day | $\bar{X}-1$ S.E. (n=4) | Size \bar{X} (n=4) | $\bar{X}+1$ S.E. (n=3) |
|-----------|---------------------------|----------------------------|---------------------------|
| 1 | 3 | 3 | 3 |
| 2 | 3 | 3 | 3 |

b. Eggs obtained from batch females imagoes.

| Hatch day | Duration | |
|-----------|----------|-----|
| | 40d | 80d |
| 1 | 6 | 6 |
| 2 | 6 | 6 |
| 3 | 6 | 6 |
| 4 | 6 | 6 |

in individual petri dishes at room temperature to hatch. To ensure that differences in time to hatching were due to endogenous differences and not exogenous differences (e.g., oxygen, temperature), all petri dishes containing eggs were individually aerated. All petri dishes were confined to a small area on a countertop, to ensure uniform temperature and lighting. Therefore, any differences in hatching time were attributable to endogenous factors (genetic, energy content of eggs etc.).

One set of eggs failed to hatch, reducing the number of replicates to 11. The female imagoes were chosen on the basis of size (body length) so that the mean body length \pm 1 standard deviation were represented: four female imagoes were 18.5 mm (mean length) three were 21 mm (mean + 1 S.D.), and four imagoes were 16 mm (mean - 1 S.D.). The mean length \pm 1 S.D. was based on measurements obtained from the female imagoes collected for individual egg collections on 4 and 6 July 1989 (Fig. 1.2).

The eggs were monitored daily for hatching. Seven larvae were added to each of three replicate tanks (500 mL) on the day that larvae were first observed in a petri dish (these individuals were designated day 1 larvae). All remaining larvae were then removed from the dish and discarded. The unhatched eggs were kept at room temperature, and the next day seven newly-hatched larvae were added to three replicate tanks (designated day 2 larvae). In addition to designating larvae as day 1 and day 2, the actual number of days required for

hatching was monitored (= days of incubation). Tanks were inoculated with larvae on 25 July to 4 August 1989 (depending upon day of egg hatch).

After 40 d, tanks were harvested and larvae were enumerated. This part of the pilot study was designed to demonstrate the influence of maternal parent, maternal parent size and the first two hatching days on larval size (head width, body length) and mortality after 40 d growth.

Multiple linear regression was conducted to determine those variables that were most highly correlated with mean larval size and survivorship. Maternal size, day of egg hatch and days of incubation, and their quadratic terms were independent variables included in analysis.

b. Batch Females

The second aspect of the pilot study examined the relationship between hatching time (the first four days) and survival and size of larvae reared from batch-collected females (Table 1.7b). Methodology followed that described above for single females. Batch eggs collected from females on 4 July 1989 were placed in each of four petri dishes. The larvae that appeared on the first, second, third and fourth day of hatching were apportioned among 12 replicate tanks (500 mL). The actual number of days of incubation required for egg hatch was also recorded and examined as a variable potentially contributing to size/survivorship of larvae. Six replicates were examined after 40 d. The remaining larvae were allowed

to develop for 80 d. Multiple regression analysis was used to determine the influence of these factors (day of egg hatch, days of incubation) on larval survival and mean larval size over 40 and 80 d.

(ii) Main Study

Results of the pilot study provided guidelines for appropriate sample size and replication numbers for the main study of maternal size influences on larval survival and growth. Protocols for egg hatching, inoculation and rearing in the main study closely followed the techniques described under the pilot study. However, since there was no significant effect of day of hatching on size/survivorship of larvae (see results for Pilot Study), this variable was not included in the main study. The actual time required for eggs to hatch did significantly influence size/survivorship of larvae, and therefore this factor was monitored for this study.

Forty females were selected from the individual-female collections of summer 1989 for the main maternal study. Eggs from five females belonging to each of 8 1-mm size classes (body length, 15-22 mm) were removed from cold storage. Eggs from 60 females had been set out to hatch to ensure that in the case of no hatching of eggs from some females, replicate numbers would not differ from the experimental design.

On the second day after hatching had begun larvae were transferred into plastic 1-L rearing tanks containing aerated

water and 3 cm depth of STND-1 sediment. Larvae were introduced into rearing tanks on the second day of hatch so that replicates would include both day 1 and day 2 larvae. Therefore, the results of this study are more general since they would apply to both days' hatch. Six tanks were each inoculated with 7 larvae from a single female. Larvae that had hatched and were not required for this experiment were preserved and measured (see above, Newly-Hatched Larvae).

Three tanks per female were maintained for 60 d. The remaining three tanks per female were harvested after 120 d. In all, a total of 240 rearing tanks were used in this experiment (2 time periods x 3 replicates per female x 5 females per size class x 8 size classes).

Results

A. Egg Size

(i) Pilot Study

There was significant individual-to-individual variation in mean egg size (nested ANOVA, $p < 0.001$). Simple linear regression showed that female size was positively related to egg size ($R^2 = 0.14$, $P < 0.01$, $N = 41$) whereas collection date (weeks after first emergence; 29 June = week 1) was negatively related to egg size (egg size (μm^2) = $66.618 - 4.115 \times [\text{weeks after first emergence}]$; $R^2 = 0.13$, $P < 0.02$, $n = 41$). When both factors were simultaneously considered (multiple regression), collection date was the only significant factor. However, because female size and

emergence date are themselves autocorrelated, the true cause of the variation in egg size is unclear. Further measurements and analyses were examined in the main study.

(ii) Main Study

A nested ANOVA revealed that there was no significant variation among dates ($P > 0.05$; Table 1.8), but there was significant variation among maternal sizes ($P < 0.01$) and among females ($P < 0.001$).

However, a stepwise forward linear regression indicated that date was the only factor significantly influencing egg size (d.f.= 1, 127; $F = 5.42$; $P < 0.025$) although the amount of variation explained was very low ($R^2 = 0.045$).

Because the majority of female imagoes in the study were H. limbata and not H. rigida (108:20) and since H. rigida females were clustered in the two middle dates (7 July and 21 July 1990), the data were reanalyzed using only H. limbata eggs.

A nested ANOVA, using H. limbata eggs only, indicated that there was significant variation among date ($P < 0.05$; Table 1.9), female size ($P < 0.005$) and among females ($P < 0.001$).

A stepwise forward linear regression revealed that date was the only factor significantly influencing egg size (d.f.=1, 107; $F = 6.79$; $P < 0.025$); again, little variation in egg size was explained ($R^2 = 0.06$). Imagoes that emerged early in the season (7 July 1991) produced the largest eggs while imagoes that emerged later in the season (28 July 1991)

Table 1.8. Results of nested analysis of variance of effects of date, maternal size, and individual female on egg size (μm^2). Analysis included eggs of *H. limbata* and *H. rigida* females.

| <u>Source of Variation</u> | <u>Degrees of Freedom</u> | <u>Sum of Squares</u> | <u>F</u> | <u>p</u> |
|----------------------------|---------------------------|-----------------------|----------|----------|
| Date | 3 | 619016.5 | 2.16 | n.s. |
| Female Size | 23 | 2231130.7 | 2.12 | <0.01 |
| Among Females | 100 | 4574185.2 | 40.09 | <0.001 |
| Within Females | 3712 | 4235320.7 | | |
| Total | 3839 | 11718072.0 | | |

Note: ns, not significant at $P = 0.05$.

Table 1.9. Results of nested analysis of variance of effects of date, maternal size, and individual female on egg size (μm^2). Analysis included eggs of *H. limbata* females only.

| <u>Source of Variation</u> | <u>Degrees of Freedom</u> | <u>Sum of Squares</u> | <u>F</u> | <u>p</u> |
|----------------------------|---------------------------|-----------------------|----------|----------|
| Date | 3 | 785466.2 | 2.69 | <0.05 |
| Female Size | 22 | 2246652.8 | 2.27 | <0.005 |
| Among Females | 80 | 3599616.6 | 41.22 | <0.001 |
| Within Females | 3132 | 3418819.2 | | |
| Total | 3239 | 10141191.0 | | |

produced the smallest eggs (Fig. 1.5).

Although egg size did vary among female sizes, there is no clear relationship between egg size and maternal size (Fig. 1.6).

B. Newly-hatched Larvae

Results of a nested ANOVA did not reveal variation in mean HW of newly-hatched larvae among maternal size classes ($P > 0.05$; Table 1.10), or among females ($P > 0.05$). The range of mean HW of newly hatched larvae was limited ($30.4 \pm 0.980 \mu\text{m}$ to $33.6 \pm 0.980 \mu\text{m}$).

Although significant variation in BL among the maternal size classes was not detected ($P > 0.05$; Table 1.11), there was variation among females within classes ($P < 0.001$). The mean larval BL ranged from $0.81 \pm 0.013 \text{ mm}$ to $0.96 \pm 0.011 \text{ mm}$. Therefore, although there are differences in size of newly-hatched offspring among female imagoes, these size differences are not related to maternal size.

C. Size/Survivorship of Offspring

(i) Pilot Study

a. Individual Females

A step-wise forward multiple linear regression was used to indicate those variables that were most highly correlated with mean size (HW) of larvae per rearing tank after 40 d growth. The regression indicated that of all variables examined (maternal size, day of egg hatch, days of incubation,

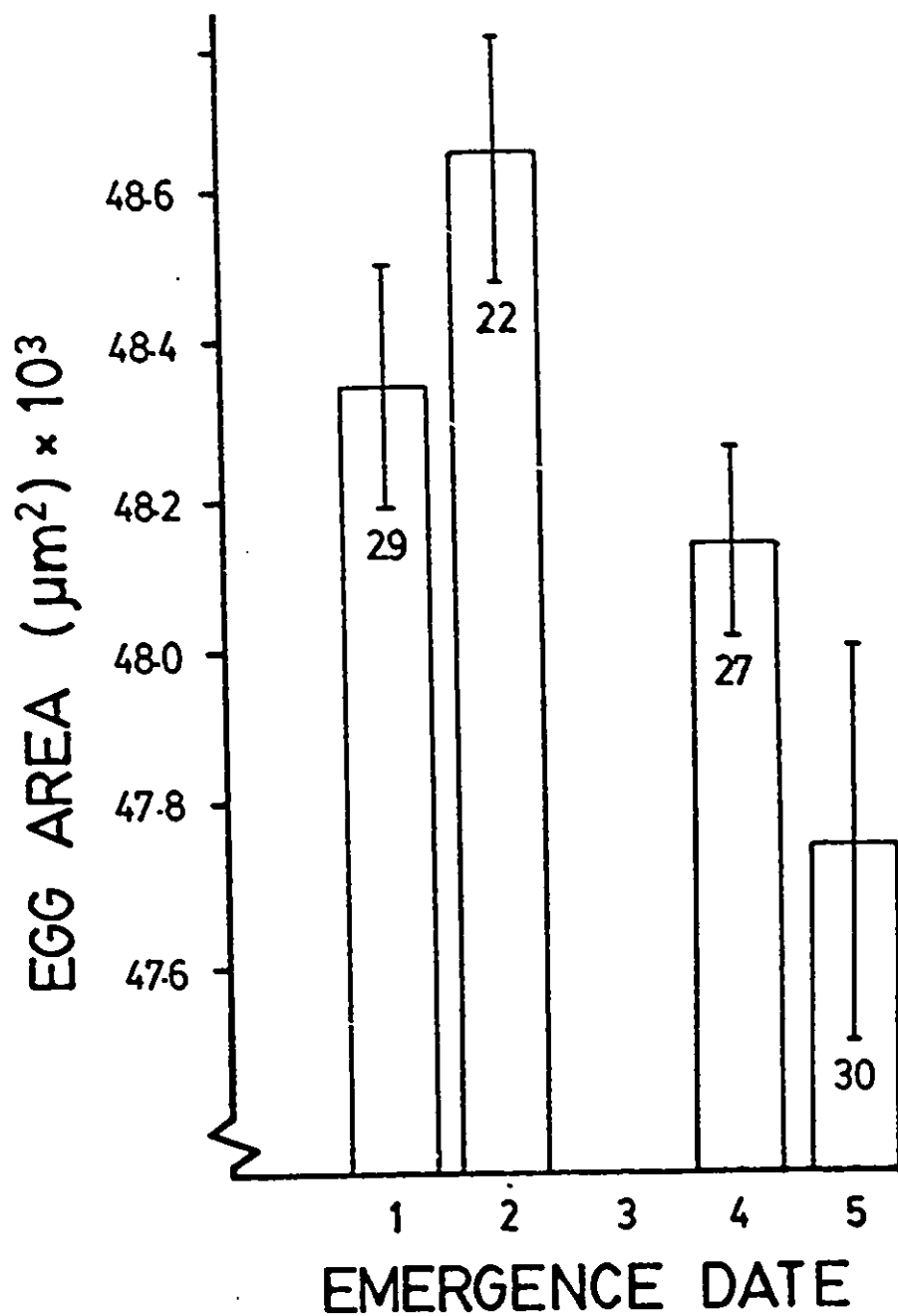


Fig. 1.5. Mean (± 1 S.E.) egg area (μm^2) for females collected throughout the emergence period in 1990. Emergence dates correspond to weeks in collection (1= 29 June, 2= 7 July, 4= 21, 23 July, 5= 28 July. No female imagoes collected during week 3). Numbers associated with error bars indicate number of females per collection date.

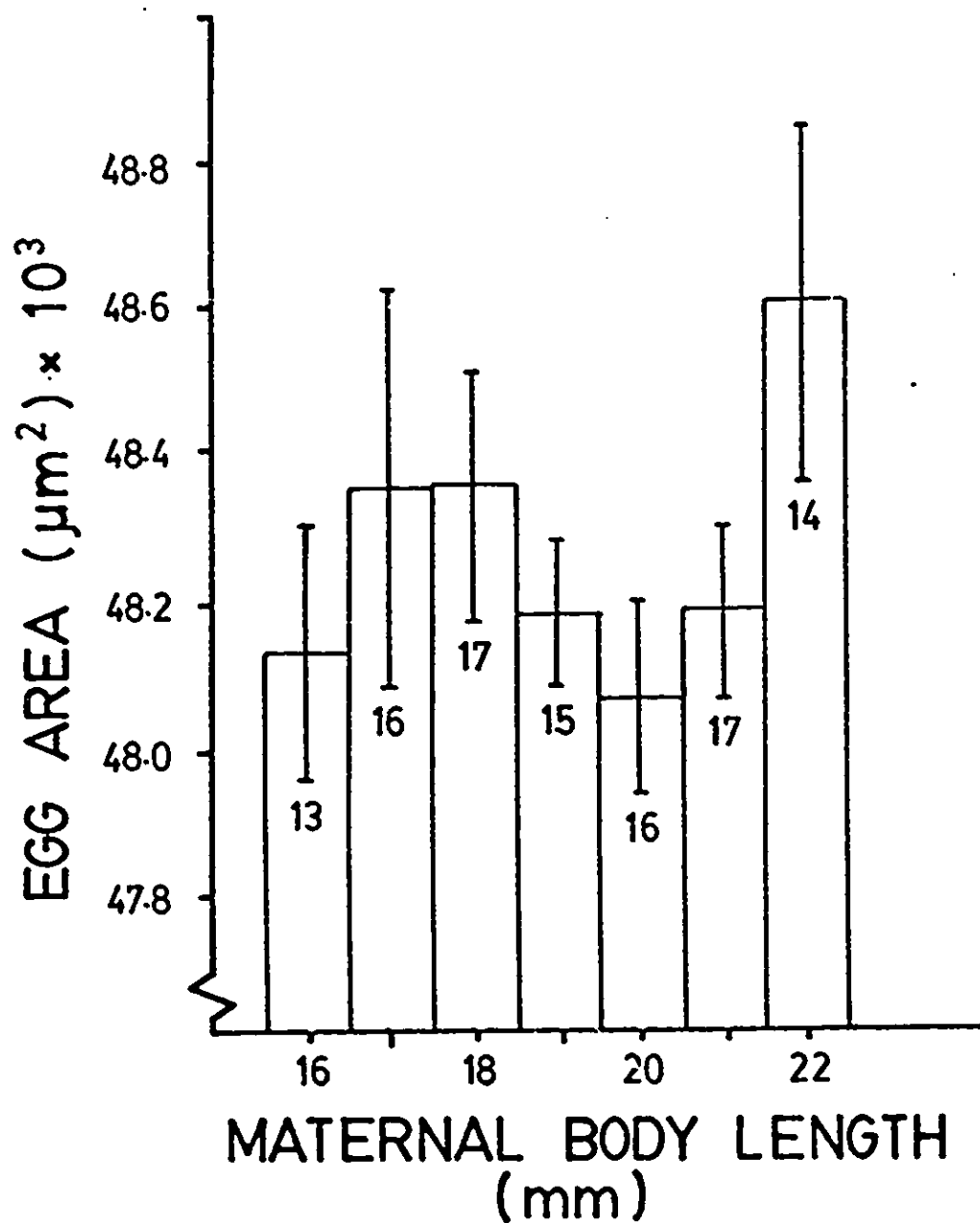


Fig. 1.6. Mean (± 1 S.E.) egg area (μm^2) among females of different sizes (body length, mm). Numbers associated with error bars indicate number of females per size class.

Table 1.10. Results of nested analysis of variance of effects of maternal size, and individual female on head width of newly-hatched larvae.

| <u>Source of Variation</u> | <u>Degrees of Freedom</u> | <u>Sum of Squares</u> | <u>F</u> | <u>p</u> |
|----------------------------|---------------------------|-----------------------|----------|----------|
| Size Class | 7 | 1.02 | 0.88 | n.s. |
| Among Females | 32 | 5.28 | 0.90 | n.s. |
| Within Females | 160 | 29.20 | | |
| Total | 199 | 35.50 | | |

Note: ns, not significant at $P = 0.05$.

Table 1.11. Results of nested analysis of variance of effects of maternal size, and individual female on body length of newly-hatched larvae.

| <u>Source of Variation</u> | <u>Degrees of Freedom</u> | <u>Sum of Squares</u> | <u>F</u> | <u>p</u> |
|----------------------------|---------------------------|-----------------------|----------|----------|
| Size Class | 7 | 62.72 | 0.30 | n.s. |
| Among Females | 32 | 948.98 | 11.25 | <0.001 |
| Within Females | 160 | 421.60 | | |
| Total | 199 | 1433.28 | | |

Note: ns, not significant at $P = 0.05$.

per cent larval survival), per cent larval survival was most highly correlated with the mean larval size in a tank ($P < 0.001$; Table 1.12). Maternal size was also significantly negatively correlated with larval size ($P < 0.01$). With increasing maternal size, larvae are subsequently smaller after 40 d larval growth (Fig. 1.6).

Linear regression was also used to determine which variables significantly influenced per cent survival of the larvae (Table 1.13). Maternal size was most highly correlated with per cent larval survival ($P < 0.02$). Maternal size² also was significant ($P < 0.02$). Maternal parents of the mean maternal size (18.5 mm, $n=49$) produced larvae with the highest survivorship (ca. 89%) after 40 d larval growth (Fig. 1.6). The length of incubation (days) (which differs from day of egg hatch) required for egg hatch was negatively associated with per cent larval survival ($P < 0.001$). Larvae that required fewer days to hatch had higher survivorship than larvae requiring a longer period of time to hatch.

b. Batch Females

After 40 d growth, none of the variables examined (hatch day, day of incubation) was significantly correlated with larval size or survivorship (multiple regression, $P > 0.05$).

After 80 d growth, the length of incubation required for egg hatch (not day of hatch) significantly influenced larval size ($P < 0.05$; Fig. 1.8). The longer the time required for eggs to hatch, the larger the resulting larvae. None of the variables examined significantly influenced larval

Table 1.12. Regression coefficients and coefficients of determination of variables significantly influencing head width of larval Hexagenia after 40 d growth.

| VARIABLE | INTERCEPT | REG. COEFF. | S.E. | R ² |
|--|-----------|-------------|-------|----------------|
| | 24.729 | | | |
| ($\frac{1}{2}$ SURVIVAL) ² | | 0.006*** | 0.001 | 0.259 |
| MATERNAL SIZE | | -0.579** | 0.190 | 0.108 |
| TOTAL | | --- | --- | 0.368 |

*** P < 0.001

** p < 0.01

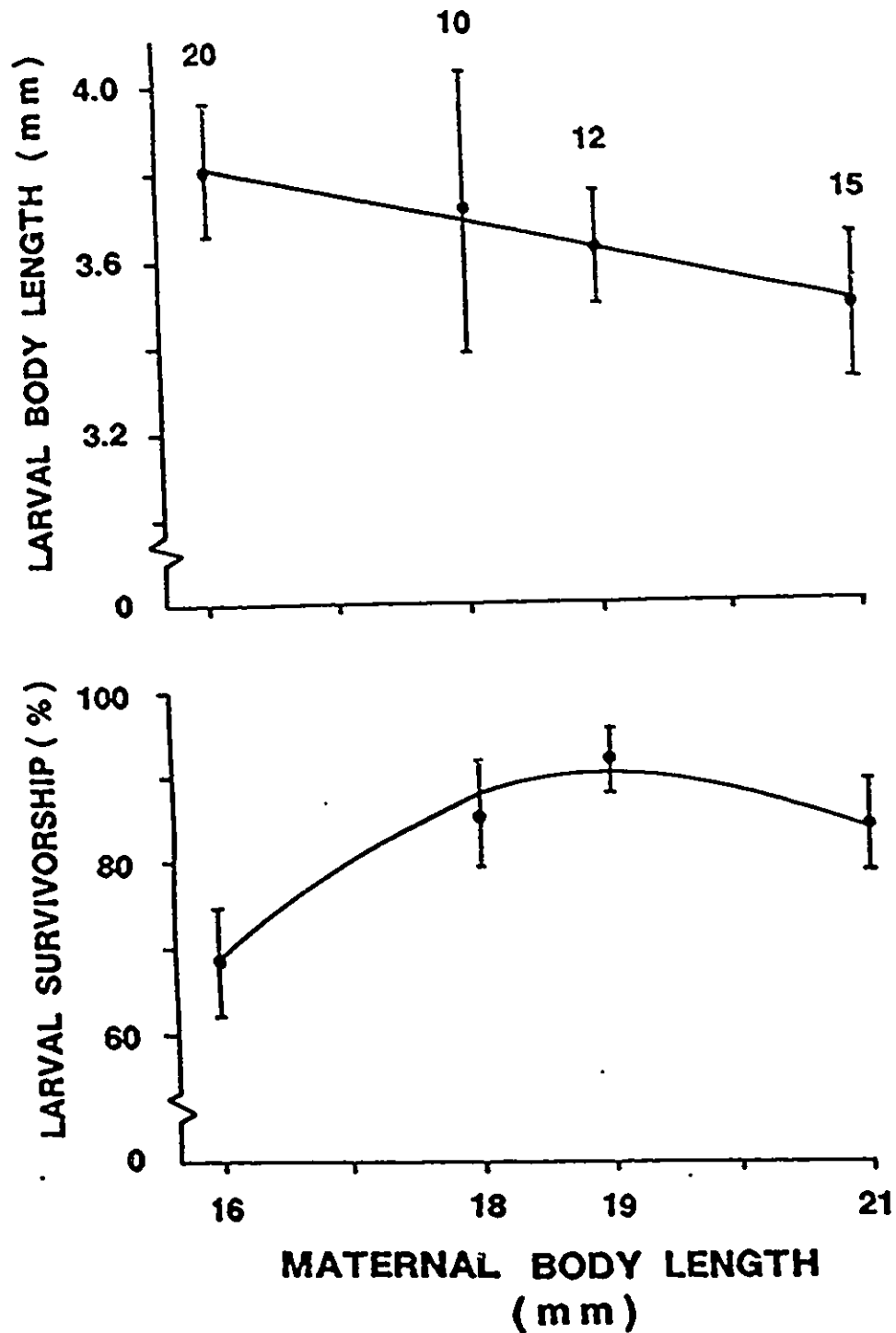


Fig. 1.7. Regression of maternal size (body length, mm) and mean (± 1 S.E.) larval size (body length, mm) and survivorship (per cent). Each variable was regressed independently with maternal size. Numbers associated with error bars indicate number of tanks used in analysis.

Table 1.13. Regression coefficients and coefficients of determination of variables significantly influencing percent larval survival after 40 d growth. (INC. = incubation)

| VARIABLE | INTERCEPT | REG. COEFF | S.E. | R ² |
|------------------------------|-----------|------------|--------|----------------|
| | -733.072 | | | |
| MATERNAL SIZE | | 92.046** | 35.801 | 0.080 |
| LENGTH OF INC. | | -9.690*** | 3.250 | 0.105 |
| (MATERNAL SIZE) ² | | -2.387** | 0.970 | 0.084 |
| TOTAL | | --- | --- | --- |

*** P < 0.001

** P < 0.02

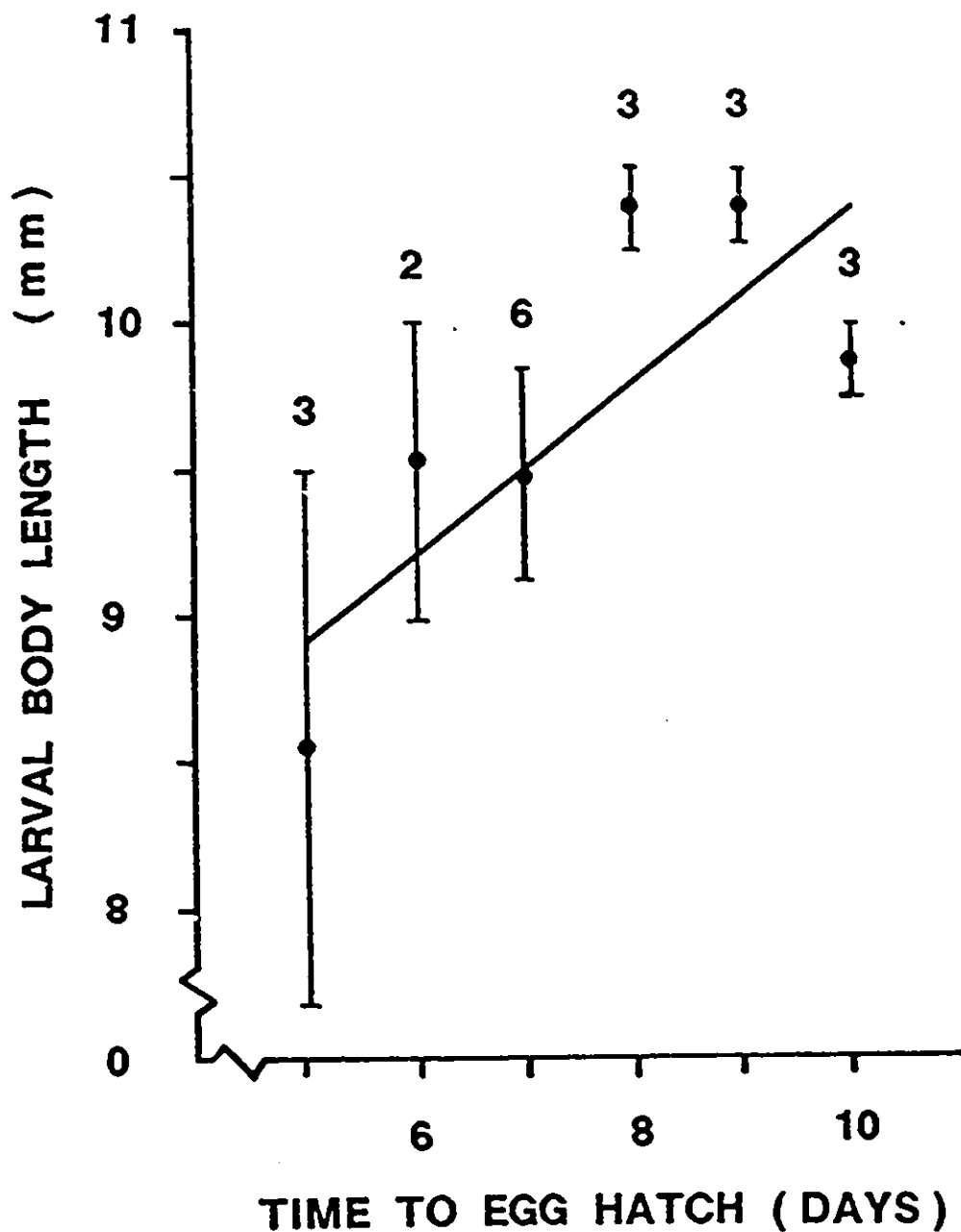


Fig. 1.8. Regression of time to egg hatch and mean (± 1 S.E.) larval size (mm) after 80 d growth. The numbers associated with error bars indicate the number of tanks used in analysis (Reg. Coeff. = 1.414, $R^2 = 0.235$, $P < 0.05$).

survivorship after 80 d growth.

(ii) Main Study

Survival in 60 and 120 d growth tanks was unusually low. In the pilot studies, 40-d larval survival ranged from 70-100% per tank. In the main study, however, complete mortality (0% survival) occurred in over 1/3 of the rearing tanks. Larval survival (proportion per rearing tank) was unrelated to either maternal size or to individual female (nested analysis of variance, $P > 0.05$; Table 1.14). Mortality was most likely caused by infestation of tanks with chironomid larvae early in the experiment and/or repairs to the airlines, which apparently introduced rust particles and toxic compounds into the growth tanks. The poor survival may have contributed substantially to the equivocal results of this study.

After 60 d growth, mean larval size ranged from 3 - 5 mm (means of means of body length per tank; Fig. 1.9). Results of the nested analysis of variance indicated that there was significant tank-to-tank variation in size (head width) of offspring of individual females $P < 0.001$ and a significant maternal size effect ($P < 0.005$), but no significant female-to-female variation ($P > 0.75$; Table 1.15). Significant differences had been observed in the pilot study also. However, the results of the main study were inconsistent with those of the pilot study. In the pilot study, larval head width after 40 d was negatively related to maternal size. In the main study, offspring of 21-mm females

Table 1.14. Results of nested analysis of variance of effects of maternal size, and individual female on larval survival (per cent) after 60 d in rearing tanks.

| <u>Source of Variation</u> | <u>Degrees of Freedom</u> | <u>Sum of Squares</u> | <u>F</u> | <u>p</u> |
|----------------------------|---------------------------|-----------------------|----------|----------|
| Size Class | 7 | 8555.83 | 1.41 | n.s. |
| Female | 28 | 23095.44 | 1.51 | n.s. |
| Within | 39 | 21323.78 | | |
| Total | 74 | 52975.05 | | |

Note: n.s., not significant at $p = 0.05$.

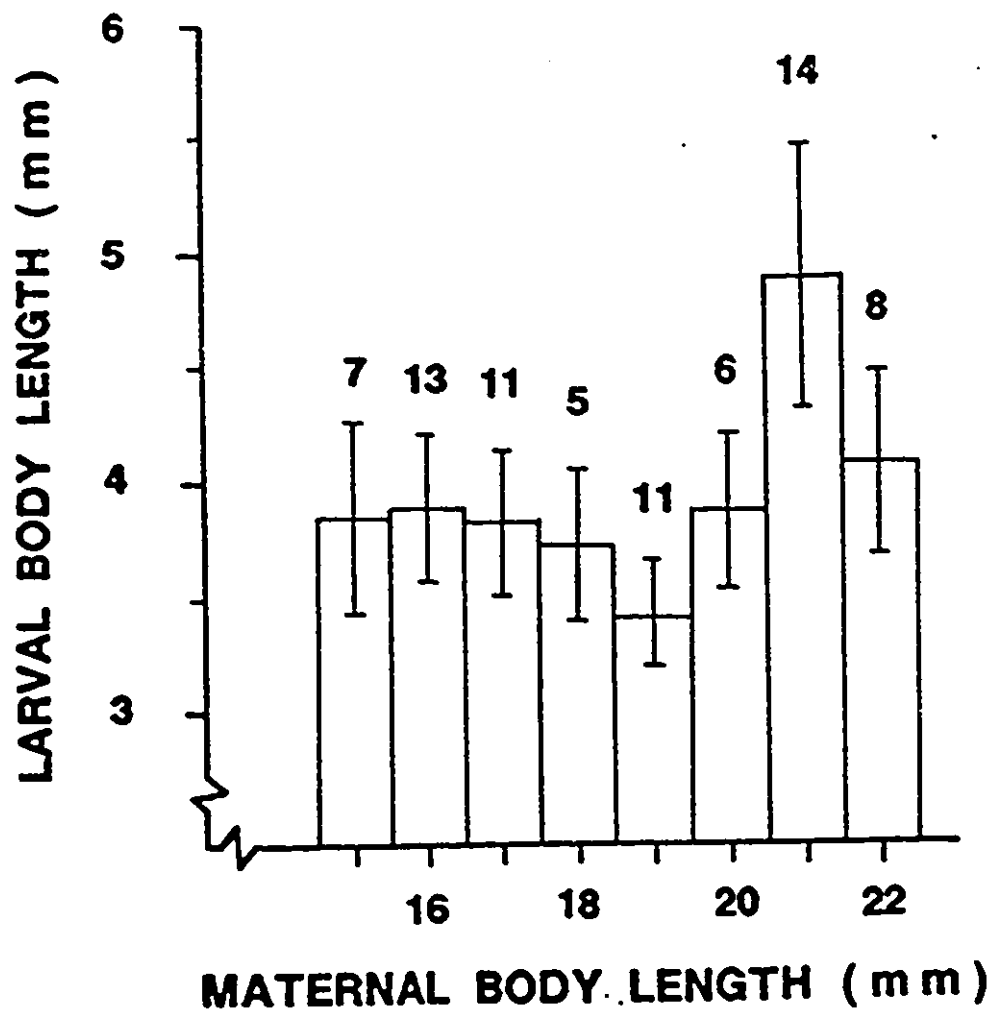


Fig. 1.9. Relationship between maternal size (body length, mm) and mean (± 1 S.E.) body length (mm) of larvae after 60 d growth. Numbers associated with error bars indicate the number of tanks used in the analysis.

Table 1.15. Results of nested analysis of variance of effects of maternal size, individual female and rearing tank on larval head width after 60 d in rearing tanks.

| <u>Source of Variation</u> | <u>Degrees of Freedom</u> | <u>Sum of Squares</u> | <u>F</u> | <u>p</u> |
|----------------------------|---------------------------|-----------------------|----------|----------|
| Size Class | 7 | 3293.46 | 2.77 | <0.025 |
| Among Females | 28 | 4751.51 | 0.63 | n.s. |
| Among Tanks | 39 | 10623.77 | 10.70 | <0.001 |
| Within Tanks | 236 | 63077.44 | | |
| Total | 310 | 24976.44 | | |

Note: ns, not significant at $P = 0.05$.

were larger than most size classes, offspring of 19-mm females were smaller than other size classes, and larvae from parents in the remainder of size classes were of equal size (Fig. 1.9).

After 120 d growth, results of nested analysis of variance indicated that there was significant tank-to-tank variation in size of offspring within individual females ($P < 0.005$; Table 1.16) but no significant maternal size effect ($P > 0.05$) and no significant female-to-female variation ($P > 0.05$). Mean larval size was 1.27 ± 0.048 mm (mean \pm S.E. of means of head width per tank) (Fig. 1.10). The HW and BL data followed the equation $BL = 3.124 + 6.790 HW$.

After 120 d growth, results of a nested analysis of variance indicated that there was no significant tank-to-tank variation in larval survivorship ($P > 0.05$; Table 1.17) nor a significant effect of maternal size effect ($P > 0.05$) on larval survivorship (Fig. 1.10). Overall mean larval survival (\pm S.E.) for tanks containing larvae was $52.10 \pm 3.651\%$ ($n = 68$).

Table 1.16. Results of nested analysis of variance of effects of maternal size, and individual female on head width of larvae at 120 d growth.

| <u>Source of Variation</u> | <u>Degrees of Freedom</u> | <u>Sum of Squares</u> | <u>F</u> | <u>p</u> |
|----------------------------|---------------------------|-----------------------|----------|----------|
| Size Class | 7 | 8189.3 | 1.20 | n.s. |
| Among Females | 20 | 21970.0 | 1.34 | n.s. |
| Within Females | 25 | 20455.0 | 2.19 | <0.005 |
| Within Tanks | 180 | 67068.0 | | |
| Total | 247 | 124957.3 | | |

Note: ns, not significant at $P = 0.05$.

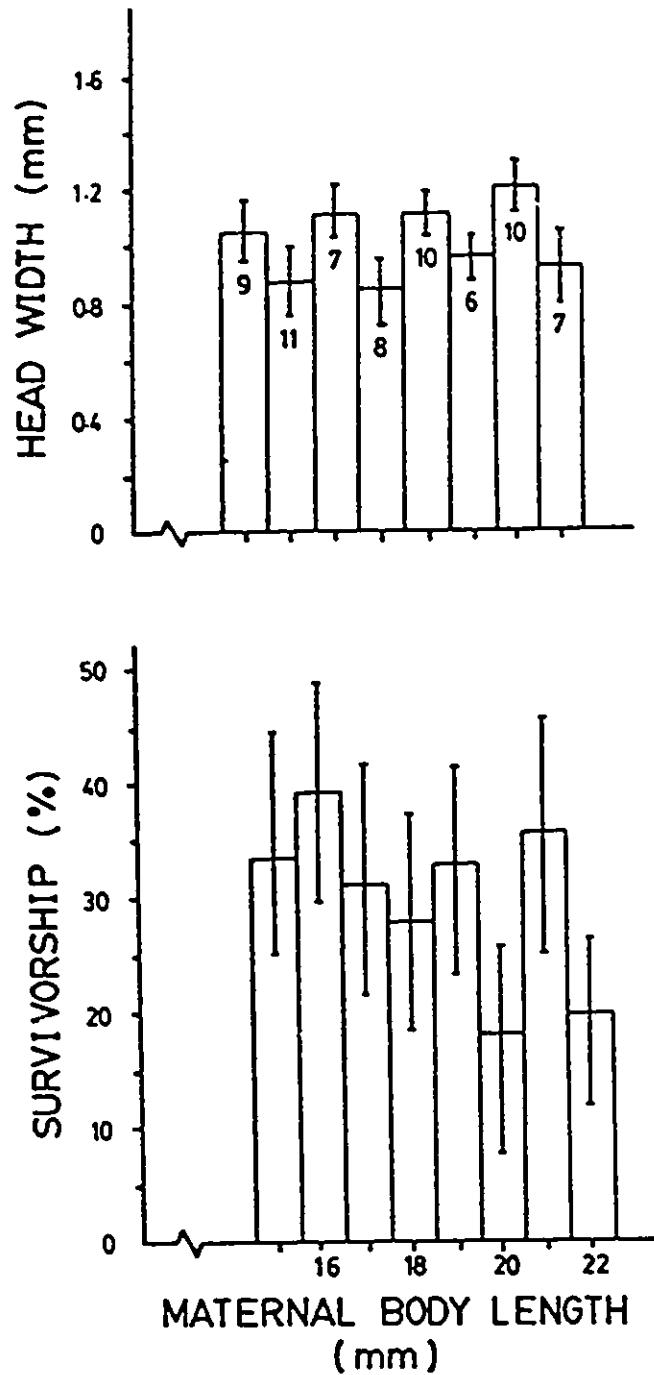


Fig. 1.10. Relationship between maternal size (body length, mm) and mean (± 1 S.E.) offspring size (head width, mm) and offspring survival (per cent). Numbers associated with error bars indicate number of tanks used in analysis.

Table 1.17. Results of nested analysis of variance of effects of maternal size, and individual female on survival (per cent) of larvae at 120 d growth.

| <u>Source of Variation</u> | <u>Degrees of Freedom</u> | <u>Sum of Squares</u> | <u>F</u> | <u>p</u> |
|----------------------------|---------------------------|-----------------------|----------|----------|
| Size Class | 7 | 6787.2 | 0.78 | n.s. |
| Among Females | 32 | 39673.6 | 1.19 | n.s. |
| Within Females | 80 | 83536.0 | | |
| Total | 119 | 129995.6 | | |

Note: ns, not significant at $P = 0.05$.

DISCUSSION

Hexagenia mayflies exhibit great variation in size in both larval and adult populations. Temperature has been considered to be one of the most important factors contributing to mayfly growth (see Sweeney 1984). However, since size variation occurs within both field and laboratory populations, factors other than temperature clearly must contribute to size differences. This study addressed the contribution of adult size variation over time, synchronization of egg hatching, presence of egg diapause and interspecific mating to size variation of Hexagenia larvae.

Variation Among Adults and Eggs

Adult mayflies were collected in the summer of 1989 and 1990. In 1989, adult imagoes were collected on only two occasions (4 and 6 July). Male imagoes consisted of H. limbata and H. rigida in a 2:1 ratio. In 1990, adult mayflies were collected almost weekly throughout the emergence period. H. limbata male imagoes were larger than H. rigida male imagoes. Burks (1953) reported the size of H. limbata female imagoes to be 22-24 mm in length and male imagoes 16-21 mm. H. rigida female and male imagoes were reported to have a body length of 18-28 and 18-24 mm respectively. The mean size of Hexagenia collected from Lake St. Clair is at the lower end of the published size range for this mayfly.

Imago collections in 1990 revealed that mean female size

was not consistent throughout the emergence season. Although size was not linearly related to emergence date, mean female size tended to decrease throughout the emergence period.

Corkum (1978) observed a decrease in size of Paraleptophlebia mollis adults at the end of the emergence period. She hypothesized that warm temperatures at the end of the summer forced small larvae to emerge. Previously, Coleman and Hynes (1970) indicated that 4 of the 11 observed mayfly species present in a southern Ontario stream demonstrated a gradual decrease in size of adults throughout the emergence period. The remaining seven mayfly species either showed no decrease in size, or sample sizes were too small to detect a change in adult size. In many mayfly species with an extended emergence period, early emerging individuals tend to be larger than those emerging later (Clifford and Boerger 1974). A decrease in female size over the emergence period may influence the ultimate number and/or size of eggs (see maternal size discussion).

In this study, determining the true relationship between female imago size and emergence date may be confounded by the presence of two species at the collection site. Differences in imago size and emergence patterns between the species may obscure the relationship. If one assumes that the ratio of H. limbata to H. rigida male imagoes accurately reflects the species ratio of female imagoes, then the species ratio of female imagoes is not constant over the emergence period. The absence of H. rigida males on the last two collection dates

suggests a preponderance of H. limbata adults, and this could explain why mean body length of female imagoes apparently increased during the last two weeks of emergence (since H. limbata adults are larger than H. rigida adults).

It is not clearly understood why female imago size decreases over the emergence period. For female mayflies it may be advantageous to be large so that many offspring are produced. Hunt (1953) calculated fecundity of 137 eggs for every 1 mm body length of H. limbata. Edmunds et al. (1976) stressed that the sole function of mayfly adults is to reproduce and that every adaptation has been directed towards this process. As mayfly body size decreases during the emergence season, a correlated decrease in fecundity was observed (Clifford and Boerger 1974, Boerger and Clifford 1975). Even if full reproductive development has not been achieved near the end of the emergence period, it may be more advantageous for a female to emerge and reproduce, than to risk another year of predation. Clifford (1969) suggested that for the mayfly Leptophlebia cupida (Say) there was a time when larvae reached a 'mature' stage at which they could transform to subimagoes regardless of size. How long the larvae remain in this 'mature' stage before transforming, and hence continue to grow, depends on environmental factors. Photoperiod and/or temperature are environmental cues that probably indicate when to emerge (Corkum and Hanes 1992). An advantage to reproducing one year earlier may offset the cost of producing a smaller clutch size. The earlier an organism

reproduces, the higher the resulting population growth (Begon and Mortimer 1986).

I observed little variation in male imago size over the emergence period. Perhaps small size in male imagoes is selected for greater maneuverability in mating swarms (Corkum 1987). McLachlan (1986) hypothesized that small size in male insects may be selected for increased agility in the swarm, resulting in increased mating events. Indeed, he did find a discrete class of very small males in copula that was absent in the unmated swarm in two chironomid species. Perhaps, since small male size is selected for, and there may be no trade-off between rapid maturation and reproductive value in males, there is a resulting constancy in male image size throughout the emergence period.

Egg Hatch and Diapause

I monitored hatching of eggs collected from female imagoes in 1989 to determine per cent viability. Eggs that were kept at room temperature began to hatch approximately two weeks after collection, and complete hatching (>95%) occurred within four days. Typically, eggs of Hexagenia mayflies do hatch over a short time span (<one week), although the time to first hatching does vary (Hunt 1953, Flattum 1963, Friesen et al. 1979, Giberson and Rosenberg 1992b, Corkum and Ciborowski 1993), and is temperature dependent.

The influence of cold storage on egg hatch was also examined. Eggs that were kept in cold storage for six months

(26 weeks) exhibited high viability when returned to room temperature. Freisen et al. (1979) found no decrease in hatching success of Hexagenia eggs until after 41 and 52 weeks of cold storage, at which time viability was reduced to 88.9 and 71.8 per cent, respectively. Flattum (1963) and Fremling (1967) observed a similar reduction in per cent egg hatching and larval vitality with time. However, Giberson and Rosenberg (1992b) found that longer time intervals in cold storage generally improved per cent egg hatch, although this result may be complicated by an egg diapause stimulated by cold storage (see below). Therefore, eggs can be maintained for long periods of time and still exhibit excellent viability.

I also tested for the presence of an egg diapause. Eggs that require cold temperature to stimulate hatch when returned to room temperature are determined to have an obligate egg diapause whereas eggs that do not require cold temperature to stimulate hatch at room temperature, but are placed in cold temperature for storage, are considered dormant. Very few of the eggs (< 0.01 %) that originally did not hatch at room temperature, hatched when returned to room temperature after four months of cold storage. Therefore, it appears that there is not a required diapause for eggs of Lake St. Clair Hexagenia. However, there is anecdotal evidence suggesting that Lake Erie Hexagenia may have an obligate egg diapause. Britt (1955) allowed eggs from one female to hatch at room temperature for two months, then placed them in cold storage

for 1.5 months before returning them to room temperature at which time 90 additional eggs hatched. Egg diapause has been observed in some populations of the mayfly Ephemerella ignita but not others (Elliott 1978). Although I did not detect diapause for Lake St. Clair Hexagenia (42°20'N), Giberson and Rosenberg (1992b) report that about 27 per cent of Hexagenia eggs collected from Southern Indian Lake, Manitoba (57°40'N) imagoes require cold storage to hatch. Possibly, at this northern latitude with its short summer season, it is advantageous for mayflies to have an egg diapause so that larvae hatch in spring rather than early autumn. Eggs are more likely to survive unfavourable conditions (e.g. winter conditions) than small larvae (Hynes 1970, Giberson and Rosenberg 1992a).

Although there is not a mandatory egg diapause in Lake St. Clair Hexagenia, overwintering in the egg stage may still occur if eggs are oviposited late in the season. Thomforde and Fremling (1968) detected small larvae in laboratory tanks three months after initially adding Hexagenia eggs; control eggs, maintained in petri dishes outside of the aquaria, had hatched two months previously. Thomforde and Fremling (1968) hypothesized that low water temperatures in the aquaria caused the delay in egg hatch. Heise et al. (1987) detected the presence of small Hexagenia larvae at the beginning of the summer in Dauphin Lake, Manitoba, possibly resulting from eggs that had overwintered. Occurrence of early instar larvae in Lake St Clair, at the beginning of the warm season, prior to

adult emergence, would support the hypothesis that delayed recruitment of larvae by overwintering of eggs does occur. To detect the presence of these small larvae, sampling with a small (200 μ m) mesh size would be required (Heise et al. 1987).

Artificial Insemination

The artificial insemination of eggs in 1990 met with limited success. Friesen et al. (1979) indicated that artificial insemination was an unreliable source of viable eggs. Giberson (University of Prince Edward Island, personal communication) also reported that the success of artificially inseminating H. rigida was 'hit or miss'.

When the artificially inseminated eggs only hatched in one species treatment (e.g., H. limbata) and not the other (e.g., H. rigida), I concluded that the species of the female imago was consistent with the species of the male whose sperm successfully fertilized the eggs. Thus, ten of the females that were artificially inseminated were determined to be H. limbata whereas only 1 female was H. rigida. This suggests that H. limbata females were more numerous than H. rigida females. However, on the dates that the female subimagos were collected for artificial insemination, H. limbata did not dominate the male subimago/imago collection. Accordingly, either the species ratio of the collection does not reflect the conclusions of the artificial insemination experiment or H. rigida females' eggs did not respond well to artificial

insemination. However, Giberson (University of Prince Edward Island, personal communication) has successfully artificially inseminated H. rigida females using the same method.

When parthenogenic eggs apparently did hatch (i.e., when no hatch occurred in either species cross), viability of the eggs was generally less than 5 per cent (although 20% hatching was observed in the unfertilized treatment in two females). Incidence of parthenogenic Hexagenia eggs is reported to be less than 5 per cent (Freisen 1981). Even in mayfly populations where males comprise a small percentage (ca. 20 %) of the adult population, when examined, the majority of females collected from the swarm are found to contain sperm on their eggs (Ephoron shigae, Watanabe et al. 1989). It is unlikely that parthenogenesis is an important factor in natural Hexagenia populations.

When hatching was observed in all three treatments (H. limbata, H. rigida, and unfertilized), the implication is that the females had previously been mated. However, the adult females were collected as subimagos, and there are no references to Hexagenia mating as a subimago. Although the subimagos that I collected for insemination often moulted to imagoes prior to insemination, it is unlikely that mating would have occurred in the laboratory. Hexagenia mayflies require elaborate mating behaviour involving swarming, which is not considered possible under laboratory conditions (Hunt 1953, Thomforde and Fremling 1968). To determine if Hexagenia female subimagos are mating in the field, it would be

necessary to collect female subimagos, and to remove their eggs to check for the presence of sperm (Watanabe et al. 1989). Alternatively, one could monitor the hatching of field-collected subimago eggs; complete viability would indicate that eggs were fertilized, since only a small percentage of eggs are parthenogenic.

Influence of Maternal Size on Size of Eggs, Newly-Hatched Larvae and Size/Survivorship of Offspring

I examined the influence of maternal size on egg size, size of newly-hatched larvae and size/survivorship of offspring in Hexagenia. Few studies address the influence of maternal size on these characteristics in insects (Parker and Begon 1986, Mousseau and Dingle 1991), despite the fact that maternal effects have the potential to alter population growth by changing parameter values for life history traits which are critical to growth (Rossiter 1991).

A positive correlation between adult size and fecundity has been characterized for many mayflies (Brittain 1982). For Hexagenia limbata, an estimate of 137 eggs per mm body length has been established (Hunt 1953). In this study, I did not measure egg number, but assumed that, as previously demonstrated, egg number increases with female body length (Boerger and Clifford 1974).

Egg Size

As a measure of egg size, I used the visible area of eggs

mounted on microscopic slides. Gribben and Thompson (1990) found that identical conclusions could be drawn using either egg weight or egg length for the damselfly Pyrrhosoma, although there was no correlation between egg length and weight. Although egg weight is probably a better indication of overall size, visible area is a more practical variable to determine due to the small size of Hexagenia eggs, and the large number of eggs I needed to measure.

Egg size varied among emergence dates, female sizes and among different females. Egg size was found to be linearly related to sample date; egg size decreased after the first week of emergence. Gribbin and Thompson (1990) found that egg size of the damselfly Pyrrhosoma nymphula also decreased over emergence period. They argued that late-emerging females were competitive 'losers'. These small adults "suffered" reduced larval growth rates that resulted from competition with larger larvae. However, I feel that one cannot determine if late emerging adults are competitive losers since eggs (both Odonata and Ephemeroptera) are oviposited throughout the emergence season, and therefore, larvae are all different ages. Also, both taxa often have populations consisting of mixed univoltine and semivoltine individuals. As such, one could argue that adults emerging later in the season are 'choosing' to trade-off full reproductive value for rapid maturation, rather than risk another year of predation.

Although egg size did vary among female sizes, the relationship between these two variables is not clear.

Whereas large females produce more eggs than small females (Hunt 1953), maternal size did not influence egg size. Gribbin and Thompson (1990) found no relationship between female size and egg size once emergence date was accounted for. Although Steinwascher (1984) observed a positive correlation between female size (body mass) of mosquito Aedes aegypti and egg size, this relationship was true only for large females. There was no relationship between egg size and female size for small females. In other insect species, a relationship between female size and egg size is observed. In digger wasps, large females carry larger eggs than small females (O'Neil 1984). Egg size also was correlated with female size in all five species of parasitoid wasps studied by O'Neil and Skinner (1990).

Newly-hatched Larvae

Data on size variation of newly-hatched larvae in relation to female size is rare (Parker and Begon 1986). My data demonstrated that while body length (BL) of first instar larvae did vary among females, there was no linear correlation between female size and offspring body length. Although some females can produce first instar larvae that are larger than other females' offspring, female size is not a determining factor for first instar size. This result is consistent with the egg size data; egg size varied among females, but not among female sizes. In life history studies, egg size is usually assumed to be an important indicator of initial

offspring size (and presumably fitness; Smith and Fretwell 1974, Parker and Begon 1986). Although I do have measurements for egg size and first instar size for females of different sizes, I cannot directly correlate first instar size with egg size. First instar size was determined for female imagoes collected in 1989, whereas the egg study was conducted with females collected in 1990.

There is evidence to indicate that egg size can influence first instar size. The major constituent of insect eggs is yolk, which contains protein and nutrients necessary for development of the embryo and is related to the first instar larva (Needham et al. 1935). Some data on insect populations do indicate that large eggs give rise to large larvae, although data are not consistent. For some insects, egg size is observed to influence first instar larvae (stonefly, Capnia atra, Brittain et al. 1984; mosquito, Aedes aegypti, Steinwaser 1984). For other insects, this relationship does not exist (butterfly, Pararge aegeria, Wilkond and Pearson 1983). Alternatively, Rossiter (1991) suggests that eggs may vary qualitatively, to act upon larval size, which may not correlate with changes in egg size.

Size/Survivorship of Offspring

The studies examining the influence of maternal size on size/survivorship of offspring produced conflicting results. Unfortunately, the main maternal study had very high mortality, (almost one third of rearing tanks had 100%

mortality) which may have contributed to the ambiguous results of the study. Although the pilot study lasted for only 40 days, and larvae were still quite small (approximately one quarter of mature size), I will address these results since the survivorship was high (70-100% among treatments).

Maternal size was negatively correlated with size of 40-day-old larvae. Large females produced smaller offspring than small females. This result is indicative of a negative maternal effect; that is, the influence of maternal size is not necessarily predicted by the mother's phenotype (Rossiter 1991). Although this phenomenon is not common, it is known to exist in springtails (Janssen et al. 1988). Janssen et al. (1988) suggested that one potential adaptive force of negative maternal effects in springtails can be found in the fact that the species has two generations per year; one generation exists in the summer months and the next in the winter. Therefore, individuals encounter the same environmental conditions as their grandmother and have similar characteristics as their grandmother since a negative maternal influence is cyclic. With Hexagenia, there is no alteration of generations to account for a negative maternal effect; Hexagenia in Lake St. Clair have a two year life cycle. Alternatively, size of 40 day Hexagenia larvae may not be indicative of adult size. Perhaps large females produce larvae that grow slowly, but achieve large adult size. Likewise, small females may produce larvae that grow quickly to emergence, but achieve a small body size. Therefore,

maternal size may have a negative effect on larval growth rate, but a positive effect on size at maturity.

A small female may 'compensate' for a low fecundity if her offspring experience higher survivorship than a larger female with a higher fecundity. In my study, maternal size did influence larval survivorship. Females of the mean maternal size (18.5 mm) produced larvae with the highest survivorship (Fig. 1.7). Females that were either larger or smaller than the mean size, produced larvae with lower survivorship. Thus, a female of the mean maternal size may have a greater 'realized' fecundity than a larger female if a greater number of her offspring survive to reproduce. It would be necessary to rear offspring to maturity to determine which female size had the highest realized fecundity. Larval survivorship patterns may become equal over time.

The length of incubation required for eggs to hatch also influenced size/survivorship of larvae. Eggs that required a longer time to hatch resulted in larvae experiencing lower survivorship, after 40-day growth. After 80-day growth, the longer that eggs took to hatch, the larger the resulting larvae. Hanes and Ciborowski (1992) have clearly demonstrated that growth differences exist among larvae that differ by as little as one day's hatch. Larvae that required 7 days to hatch grew faster than larvae that required 6 days to hatch, but these slower-hatching larvae also experienced greater mortality. The mechanism for this difference in growth is not known. The factors contributing to the growth/survival

differences between larvae that required a different amount of time to hatch could include differences in sex, species, egg size or fertilized vs. parthenogenic eggs.

I know of no studies that compare the growth of insects among larvae that require a different number of days to hatch although some studies suggest reasons for asynchronous hatch in insects. Corkum and Ciborowski (1992) found that Hexagenia eggs collected from river sites hatched faster and had a more synchronous hatch than eggs collected from lake sites. Marois and Croll (1991) found that order of egg hatch was not related to egg size in the pond snail Lymnaea, but rather to the position of the embryo within the egg mass; embryos near the center of the mass hatched later than those on the periphery. Parthenogenic eggs of both Ecdyonurus spp. and Rhithrogena spp. required longer to hatch than fertilized eggs (Humpesch 1980); differences in growth rates between larvae hatching from fertilized and parthenogenic eggs were not examined.

Endogenous factors (such as maternal size, day of hatch) may be important enough early in larval development to contribute substantially to the outcome of competitive events that occur later in development when exogenous factors (density, food, sediment) are significant (Hanes and Ciborowski 1992). The fact that growth of 40- and 80-d -old larvae is influenced by endogenous factors, such as maternal size and day of egg hatch, is important. An assumption of many life history models is that offspring that begin with a size advantage do disproportionately well because the initial

size differences becomes amplified during competition (Parker and Begon 1986). Although I did not find that size of first instar larvae did vary with maternal size, growth of offspring is obviously dependent upon maternal size.

2. STANDARDIZED MATERIALS AND METHODS FOR REARING HEXAGENIA

INTRODUCTION

Hexagenia mayflies are ubiquitous in large waterbodies of North America. Historically, this mayfly has received much attention because bodies of spent adult Hexagenia can form putrid-smelling drifts that interfere with traffic (Hunt 1953, Fremling 1960, Carlander et al. 1967). Additionally, this mayfly is considered to be an important member of the aquatic food web (Neave 1932, Needham et al. 1935, Hunt 1953, Fremling 1960). Recently, Hexagenia has been proposed as an "ecosystem objective" (indicator of ecological health) for mesotrophic large lakes (Reynoldson et al. 1989). As such, this mayfly is sampled extensively in the field (Schloesser and Hiltunen 1984, Landrum and Poore 1988, Bedard 1990; Kovats and Ciborowski 1989, Schloesser et al. 1991) and studied in laboratory toxicity tests (Henry et al. 1986, Hare et al. 1989, Ciborowski et al. 1991).

At present, although protocols for rearing Hexagenia larvae are available (Fremling 1967, Freisen 1981) very little standardization of the materials required for rearing exists. Of the materials required for rearing larvae (eggs and/or larvae, aquaria, water, sediment, air source, food) sediment type and source of larvae are probably the most variable factors among laboratories.

Typically, researchers use local field-collected sediment for rearing burrowing mayflies. While this is an acceptable procedure, it makes comparing results among laboratories

difficult. Sediment collected from different geographical areas or from different locations within a waterbody may vary in particle size, organic content and contaminant burden (Bedard 1990). It is likely that time at which samples are collected also may account for variation in sediment characteristics. These factors can potentially influence larval growth because of differences in food levels and/or burrow stability. Thus, comparing results of experiments conducted in different laboratories, or at different times, is invalid.

A standard, person-made sediment with a consistent particle size distribution, organic matter, and contaminant burden, if widely used, could standardize experiments, facilitating comparison of biological and toxicological results among laboratories. A standard reference sediment also could be used as a control sediment in Hexagenia bioassay studies. Sediments that are currently used as controls are field-collected substrates that are considered to be relatively contaminant-free (Landrum and Poore 1988, D.C. Bedard, Ontario Ministry of Environment, personal communication).

Artificial substrates have been developed for use in Hexagenia toxicity tests. Artificial substrates have included an epoxy resin substrate (Fremling and Schoening 1973) and glass tubes glued onto glass plates (Henry et al. 1986). These substrates were designed to meet the thigmotactic needs of burrowing mayflies, while avoiding introduction of

contaminants. However, since Hexagenia larvae ingest sediment as their primary food source (Zimmerman and Wissing 1978), these artificial substrates can be used only in short term experiments.

Fremling (1967) described a more natural sediment that he used for rearing Hexagenia larvae. The sediment that he developed consisted of garden soil and thoroughly composted hay that was enriched with an inorganic fertilizer. Although Fremling (1967) successfully reared Hexagenia to adulthood in this sediment, to more closely represent field conditions, I feel that a standard sediment should have characteristics of a natural sediment known to support Hexagenia populations. Additionally, a more natural standard sediment could be used in bioassay experiments. A standard sediment should support larvae that grow quickly and yet still maintain high survivorship. Also, the source materials required to make the reference sediment should be broadly available.

In addition to standardizing sediments used in laboratory experiments, larvae also need to become more standardized (i.e., age and size of larvae, conditions under which larvae were reared, including sediment, food, water, temperature, etc.). Experiments are frequently designed to begin with half-grown (body length = 1 cm) larvae. These larvae are often collected from field populations (e.g., Zimmerman and Wissing 1978, Wright and Mattice 1981a, 1982; McCafferty and Pereira 1984, Hare et al. 1989) rather than having been reared from eggs (e.g., Thomforde and Fremling 1968, Sauter et al.

1991, Corkum and Hanes 1992, Giberson and Rosenberg 1992a, Hanes and Ciborowski 1992). Unfortunately, using field-collected individuals introduces numerous confounding factors. Although similarly sized larvae may be chosen, this does not guarantee constancy in age (Corkum and Hanes 1992; Hanes and Ciborowski 1992). Even when larvae are collected from one site, larvae may have migrated to that location (Hunt 1953; Wright and Mattice 1981b) and hence may not have been previously subjected to the same environmental conditions. If larvae are subsequently used for experiments, differences among larvae may contribute to substantial variation within experimental treatments. Variation within experimental treatments reduces the power to detect differences among treatments (Sokal and Rohlf 1981).

A standardized method for laboratory-rearing larvae in large numbers needs to be developed. For universal application of standardized rearing procedures, this rearing method should be a practical alternative to collecting larvae in the field.

In many laboratory experiments involving Hexagenia, larval growth is monitored. Researchers often examine the simultaneous influence of several variables on size/survivorship of larvae (Wright and Mattice 1982a, McCafferty and Pereira 1984, Giberson and Rosenberg 1992a, Corkum and Hanes 1992, Hanes and Ciborowski 1992). Determining the growth of mayflies is possible when an experiment is initiated with newly-hatched larvae, since

initial larval size is known (size of first-instar larvae is relatively constant). However, if the experiment is initiated with larvae that are half-grown, then initial size of larvae must be determined. When half-grown larvae are added to experiments, to estimate initial larval size, researchers typically measure a subset of larvae, not included in the experimental treatments. The mean larval size of this subset is used as a surrogate value to represent mean larval size in all treatments (but see Henry *et al.* 1986). However, this practice reduces the accuracy of measuring larval growth since only an estimate of larvae size (a constant) is determined for each replicate. In some replicates, mean larval size is found to be smaller at the end of the experiment than at the beginning (see Bedard 1990), suggesting that larvae are 'shrinking'. This conclusion may be falsely reached if larvae initially added to a replicate were smaller than the surrogate mean value, or if size-dependent mortality occurred. Clearly, a method of measuring larvae is required when it is not possible to measure preserved larvae.

Photographing specimens before and after experimental procedures is a possible alternative to measuring either preserved or live larvae. Photographs provide a permanent record of larval size. Although Henry *et al.* (1986) measured photographic images of *Hexagenia* larvae to determine growth, they did not report the accuracy of the method. A comparison of measurements of photographic images of larvae with measurements of preserved larvae is necessary to determine

both the accuracy and precision of measurements of photographic images.

This study was initiated to develop a standardized sediment for laboratory-rearing Hexagenia larvae. This standard reference sediment was developed to mimic the particle size distribution and organic matter of the natural sediment that was found most suitable for larval growth. A coupled laboratory experiment (Sediment Suitability: Larval size/ survival and Sediment Suitability: Selection by larvae) was conducted. Additionally, I wanted to find an efficient method of rearing larvae in the laboratory, to provide large numbers of half-grown larvae for laboratory experiments. And finally, I wished to ascertain the accuracy of using photographs to estimate larval size.

Specifically, my objectives were to include:

1. Collecting natural sediments from six sites to determine which sediment was most suitable for larval growth. Suitablility for larval growth was determined by:
 - a) Comparing larval size/surivivorsip on the sediments;
 - b) Determining which sediment larvae select when presented with several different sediment types;

2. Developing a standard reference sediment that mimics the characteristics of the natural sediment found to be most suitable for larval growth;
3. Developing a protocol for mass-rearing larvae;
4. Determining the accuracy of measuring photographic images of larvae.

METHODS AND RESULTS

I. Sediment Collection and Evaluation

Methods

A. Physical Characteristics of Sediments

Collection

To establish how natural sediments compare to a standard reference sediment, I collected sediments from six waterbodies in or near southern Ontario (Table 2.1). Sediment was collected from Anchor Bay, Lake St. Clair (ANC), Balsam Lake (BAL), Cook's Bay, Lake Simcoe (COO), Honey Harbour, Georgian Bay (HON), Lake Nicolet, located along the St. Marys River (NIC) and Saginaw Bay, Lake Michigan (SAG). Of these waterbodies, the Ontario Ministry of the Environment frequently samples benthos at Honey Harbour and Balsam Lake for Hexagenia and Elliptio mussels, respectively. The remaining four waterbodies have had historically large populations of Hexagenia. Sites at which I sampled within the waterbodies had previous records of Hexagenia populations.

At each of the six locations, sediment was collected offshore using a mini-ponar grab (20 cm x 20 cm). Because sediment characteristics can vary considerably over small areas within single waterbodies, it was necessary to collect sediments that were likely to be qualitatively suitable for Hexagenia. Sediment was considered suitable at a site whenever Hexagenia larvae were found in the sediment samples. Sediment was sieved at each site, off the side of the sampling

Table 2.1. The occurrence of Hexagenia larvae at each sediment collection site, 1989.

| Date | Location | | Latitude Longitude | Sediment (L) | <u>Hexagenia</u> |
|---------|--------------|----|------------------------|-----------------|------------------|
| 27 Aug | Honey Hbr., | ON | 44° 50' N 88° 25' W | 45 | present |
| 27 Aug | Balsam L., | ON | 44° 35' N 79° 49' W | 45 | present |
| 28 Aug | Cook's Bay, | ON | 43° 15' N 78° 29' W | 45 | absent |
| 17 Sept | L. Nicolet, | MI | 46° 23' N 84° 15' W | 45 | present |
| 17 Sept | Saginaw Bay, | MI | 44° 00' N 83° 40' W | 45 | absent |
| 20 Sept | Anchor Bay, | MI | 42° 35' N 82° 44' W | 25 | present |

boat, through a 1-mm sieve. In cases where no mayfly larvae were encountered, sediment was considered suitable for Hexagenia growth if it was silty, free of vegetation, and supported populations of Chironomus (Diptera: Chironomidae) larvae. Larvae of Chironomus spp. are often found in association with Hexagenia (E.C. Hanes, personal observation). Sediment was placed in 15-L plastic buckets, returned to the laboratory and placed into cold storage (4°C). All collected sediment was autoclaved (20 min, 120°C, 103 KPa) as soon as possible (within two weeks) following collection.

Sediment Composition

Organic matter content of the seven sediment samples (natural sediments from six sites and STND-1, a standard sediment mixture (see Chapter 1, Part II D)) was measured by loss on ignition of oven-dried samples. For each of the sediment types, four replicate sediment samples were used to determine organic content (per cent organic matter). The dry mass of each replicate sample (ca. 20 g) was determined on a balance sensitive to 0.0001 g, after being dried (105°C, 24 h) and ashed (550°C, 4 h). The organic content of each sediment was calculated.

The particle size distribution of the sediments was determined using hydrometer and sieve analysis (ASTM 1981). The distribution of particle sizes larger than 75 µm is determined by sieving, whereas the distribution of particle sizes smaller than 75 µm is determined by sedimentation

process using a hydrometer to secure the necessary data. The following methodology is a brief description of the process I used to determine particle size distribution of sediments (ASTM 1981).

Air dried sediments were carefully broken up with a mortar and pestle. Either 50 g (sediment with little sand) or 100 g (sediment with mostly sand, as determined by previous sieving) of sediment was weighed on a balance sensitive to 0.01 g and the mass recorded. A dilute dispersing agent (soap solution) was then added to the sediment at least 16 hours prior to testing to allow for complete wetting of the sediment. At the end of the soaking period, the sediment sample was further dispersed in a blender fitted with baffles. The sample was poured into a sedimentation chamber, and distilled deionized water was added to the 1000 mL volume. The contents of the chamber were thoroughly mixed for 1 min. by stoppering and repeatedly inverting the chamber. The sedimentation chamber was placed into a 20°C water bath. Hydrometer readings were carefully taken at intervals over a period of 24 hours as indicated by ASTM protocol. Afterwards, the sample was sieved through a 63 μ m sieve and the contents of the sieve recovered. The sediment recovered from the sieve was air-dried, crushed with a mortar and pestle and then sieved through a series of standard sieves (1.00, 0.50, 0.25, 0.125, 0.063 mm). The sediment remaining on all sieves was weighed and the mass was recorded.

Hygroscopic moisture was determined for each sediment type (ASTM 1981). A correction factor for the dispersing agent added to the distilled water also was determined (ASTM 1981). Calculations needed to transform the hydrometer readings and the mass measurements to sediment particle size distribution followed ASTM (1981) guidelines.

B. Sediment Suitability: Larval Size and Survival

As a measure of sediment suitability for rearing larvae, mayflies were reared in the seven sediment types (natural sediments from 6 sites + STND-1 sediment). One hundred mL of each sediment type was placed in standard size (500 mL) styrofoam rearing tanks [7 sediment types x 5 replicates/treatment = 35 tanks for 2 time periods (30 d and 90 d)]. On 17 January 1990, 7 newly-hatched Hexagenia larvae (see Chapter 1, Part II B) were added to each of the 70 rearing tanks (1000 larvae·m⁻²), equivalent to field densities (Hudson et al. 1986). Larvae were fed, maintained and harvested according to protocols outlined in detail in Chapter One. Tanks were harvested on 18 January 1990 (30 d) and 18 April 1990 (90 d).

One-way analysis of variance (ANOVA) was used to test for differences in larval survival and mean larval size among the sediment types. If significant differences occurred, a Student-Newman-Keuls (SNK) multiple comparison test was used to determine which groups did not differ from one another.

C. Sediment Suitability: Selection by Larvae

As a second measure of substrate suitability for larval rearing (see above - larval growth on different sediments for first measure), two replicate seven-day sediment choice experiments were conducted. These experiments were conducted to determine which sediments larvae select.

The choice-chambers consisted of 56 x 56 x 20 cm plexiglass aquaria (Fig. 2.1). An aluminum partitioning grid consisting of 49 (i.e., 7 x 7) cells (8 cm x 8 cm x 10 cm) was placed within the square plexiglass aquarium. Seven sediment types (natural sediment from 6 sites + STND-1 sediment) were placed within the the aluminum grid. The natural sediment had been autoclaved and kept in cold storage for approximately 18 weeks at the time the experiment began. A Latin Square design (Table 2.2) was used to arrange the sediments so that each sediment type was represented only once in a given row and a given column of a grid of blocks, and that no two blocks of the same sediment type were adjacent. However, the Latin Square design is limited in the number of combinations that can occur within a single replicate (Box et al. 1978). Accordingly, the arrangement of the sediment was changed between trials one and two (Table 2.2). All combinations of adjacent sediment types were examined by the two trials of the choice experiments.

Approximately 250 mL (ca. 5 cm depth) of sediment was added to each cell. Aerated, distilled water was added to the tanks to a depth of 13 cm over the sediment (Freisen



Fig. 2.1. Top view of the plexiglas sediment choice chamber with the aluminum partitioning grid in place. One row has been filled with sediment.

Table 2.2. The arrangement of sediment used for trial one and two of the sediment preference experiment. The arrangement followed a Latin Square design. Roman and Greek numbers indicating row and column were used to identify cell location.

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | | REPLICATE 1 | REPLICATE 2 |
|---|---|---|---|---|---|---|-----|--------------|-------------|
| A | B | C | D | E | F | G | I | A = NICOLET | STND SED |
| C | D | E | F | G | A | B | II | B = STND SED | BALSAM |
| E | F | G | A | B | C | D | III | C = COOKS | SAGINAW |
| G | A | B | C | D | E | F | IV | D = BALSAM | NICOLET |
| B | C | D | E | F | G | A | V | E = HONEY | COOKS |
| D | E | F | G | A | B | C | VI | F = SAGINAW | HONEY |
| F | G | A | B | C | D | E | VII | G = ANCHOR | ANCHOR |

1981). The walls of the plexiglass choice chamber were covered (on the outside) with black plastic to minimize effects of light on outer walls of peripheral cells. One mL of concentrated food mixture (Chapter 1, Part II C) was added to each cell and stirred into the sediment. The tank was aerated for one week prior to addition of larvae to ensure oxygen levels were high (Hanes 1989). Physical characteristics of the water were determined (pH = 8.2, conductivity = 250 $\mu\text{S}\cdot\text{cm}^{-1}$ and temperature 18 - 19°C).

The first trial began 29 January 1990 and the second replicate trial, 3 February 1990. Test organisms for these experiments had been reared on the STND-1 sediment at a density of 8 larvae per 63-cm² rearing chamber (1270 larvae-m⁻²) for six months prior to the experiment. Larvae were approximately "half-grown" (mean body length \pm 1 S.E. = 12.77 mm \pm 0.256, n = 382). Eight larvae were placed within each of 49 cells of the sediment with the partitioning unit in place. One hour after larvae were added to the sediment, the aluminum partitioning unit was removed; the partition was replaced 7 days later when larvae were retrieved (Wright and Mattice 1981b). Hexagenia larvae were recovered [by sieving (500 μm sieve) the sediments within each partition], and enumerated. Subsequently, larval size (HW) was determined.

To test the hypothesis that larvae were distributed randomly among the sediment types, a two-way analysis of variance was conducted to determine the effect of sediment and trial on larval sediment selection (i.e., number of

larvae/sediment type). Trial was included in the analysis to determine if there was a difference in larval selection between the two set ups. To determine if there was a row, column or edge effect of the grid, the number of larvae occurring within each row and column and within cells located around the periphery of each chamber was compared using a Latin Square analysis (SAS 1985). For this analysis, the sediment type was ignored.

Distribution with respect to larval size was addressed for organisms within the first replicate only. A one-way analysis of variance was conducted to determine if the mean size of larvae varied among different sediment types. The species of the larvae (Hexagenia limbata vs. H. rigida) was determined by examining head band patterns (D. Giberson, University of Prince Edward Island, personal communication) and male genitalia (Burks 1953). The larvae used in the second trial were not measured because larval size did not influence sediment selection in the first trial (see results Chapter 2, Part 1 C).

Results

A. Physical Characteristics of Sediment

The standard sediment had the largest amount of organic material (ca. 28%), whereas sediment collected from Anchor Bay and Lake Nicolet had the smallest amount of organic material (ca. 2%) (Table 2.3). Texture of organic materials differed between natural and the standard sediments. The

Table 2.3. The mean organic content (per cent organic matter) for the six natural sediment types and the STND-1. Values represent the mean and 1 S.E. amount of organic matter occurring in four replicate samples of each sediment type.

| | ANC | BAL | COO | HON | NIC | SAG | STND-1 |
|------|-------|-------|-------|-------|-------|-------|--------|
| X | 2.38 | 18.55 | 11.94 | 4.09 | 2.14 | 8.89 | 28.21 |
| S.E. | 0.096 | 0.772 | 0.131 | 0.247 | 0.761 | 0.086 | 0.176 |

standard sediment contained primarily leafy and woody material characteristic of a littoral aquatic sediment. The natural sediments were dominated by fine, amorphous organic material typical of profundal regions.

Both natural and standard sediments were similar in inorganic particle size distribution (Fig. 2.2). All sediments consisted of 30-60 per cent silt and 30-50 per cent fine sand.

B. Sediment Suitability: Larval Size and Survival

After 30 and 90 d growth, larval size and survivorship were compared among the seven sediment types (natural sediments from six sites + STND-1 sediment). The larvae within the 30-d series were too small and mortality was too high to conduct statistical analysis (Sokal and Rolph 1981).

Larval size did not differ among the sediment types when mean HW for all larvae within a replicate was used for analysis ($F_{[6,19]} = 2.439$, $0.10 > P > 0.05$). However, loss of replicates per sediment type within a trial often resulted from high larval mortality; complete larval mortality occurred in several tanks. The high mortality may have resulted from high numbers of chironomids invading the rearing tanks shortly after inoculation with Hexagenia larvae. Chironomids probably originated from drains in the laboratory.

The high mortality in the experiment resulted in too few data points to achieve significant results in this analysis (Sokal and Rohlf 1981). To increase the number of data

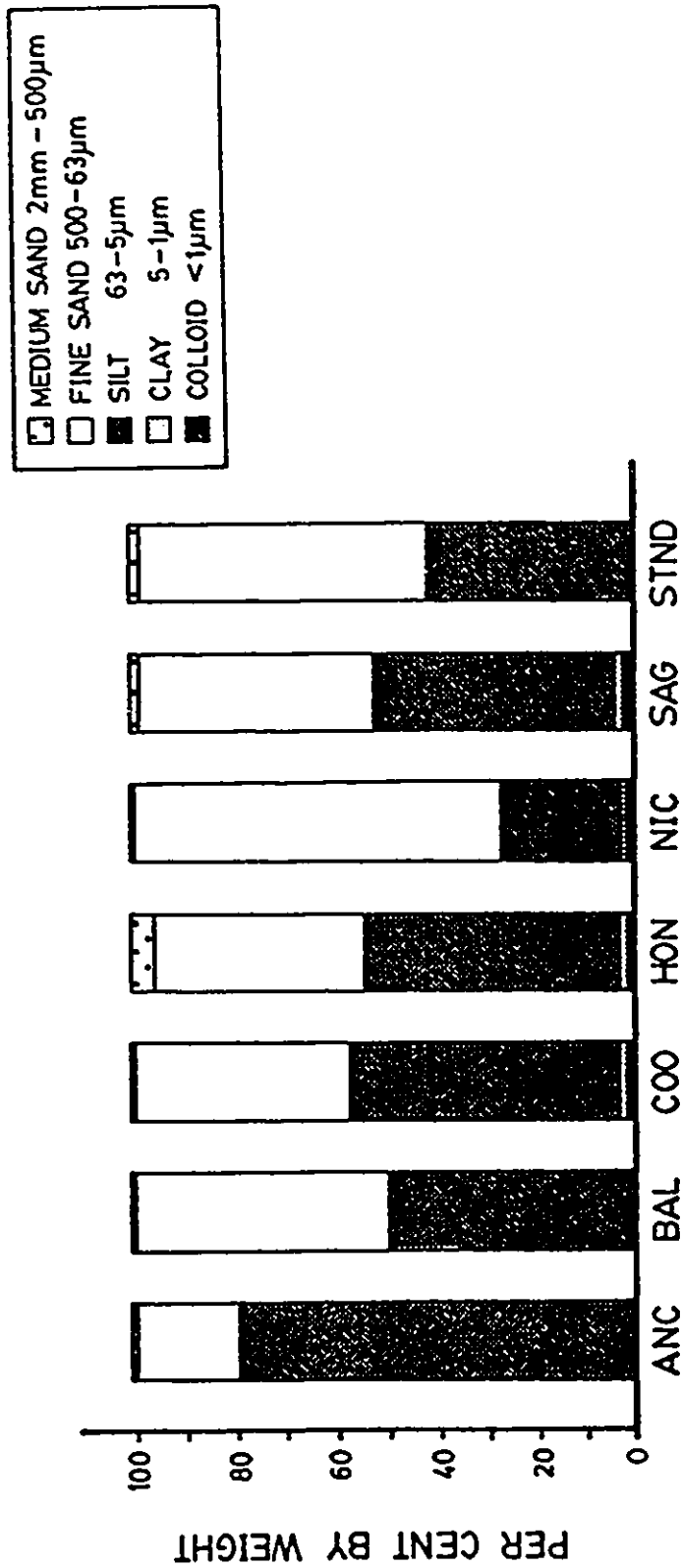


Fig. 2.2. Sediment particle size distribution for the seven sediment types (ANC = Anchor Bay, BAL = Balsam Lake, COO = Cook's Bay, HON = Honey Harbour, NIC = Lake Nicolet, SAG = Saginaw Bay, STND = standard-1). Particle sizes include medium sand (2 mm - 500 µm), fine sand (500-63 µm), silt (63-5 µm), clay (5-1 µm) and colloid (< 1 µm).

points, the data were reanalyzed using the individual HW of all larvae that survived within a particular sediment. Because there was significant variance added among replicates within treatments (Nested ANOVA, $P < 0.05$) the pooling of larvae from different replicate tanks should be treated with caution. However, using individual HW (instead of mean HW) allowed for detection of differences among sediment groups.

Larval size significantly differed among the seven sediment types (ANOVA, $F_{[6,90]} = 7.026$, $P < 0.001$; Table 2.4). A SNK test revealed the presence of four overlapping groups of sediment that varied in size of larvae that they supported ($P < 0.05$; Fig. 2.3). Smallest larvae occurred in BAL, STND-1 and ANC sediment; largest larvae occurred in HON and SAG sediment.

After 90 d growth, larval survival significantly differed among the seven sediment types (ANOVA, $P < 0.01$; Table 2.5). The SNK test detected two groups of sediment, representing high and low larval survivorship (Fig. 2.4). Overlap occurred between the two groups. Survival in SAG, NIC, COO and STND was higher than survival in ANC, BAL, HON and SAG sediment.

C. Sediment Suitability: Selection by Larvae

After 1 week in the sediment selection chamber, results of analysis of variance detected that larvae were non-randomly arranged among the cells (Table 2.6). There was a significant effect of sediment ($P < 0.001$) on larval location. Although there was no significant difference between the two

Table 2.4. Analysis of variance of larval HW among the seven types of sediment after 90 d growth. HW of all larvae occurring within a sediment type are included in the analysis.

| SOURCE OF VARIATION | df | MS | SS | F | P |
|---------------------|----|---------|--------|-------|---------|
| AMONG | 6 | 10893.9 | 1815.7 | 7.352 | < 0.001 |
| WITHIN | 90 | 22225.1 | 246.9 | | |
| TOTAL | 96 | 33119.0 | 15.7 | | |

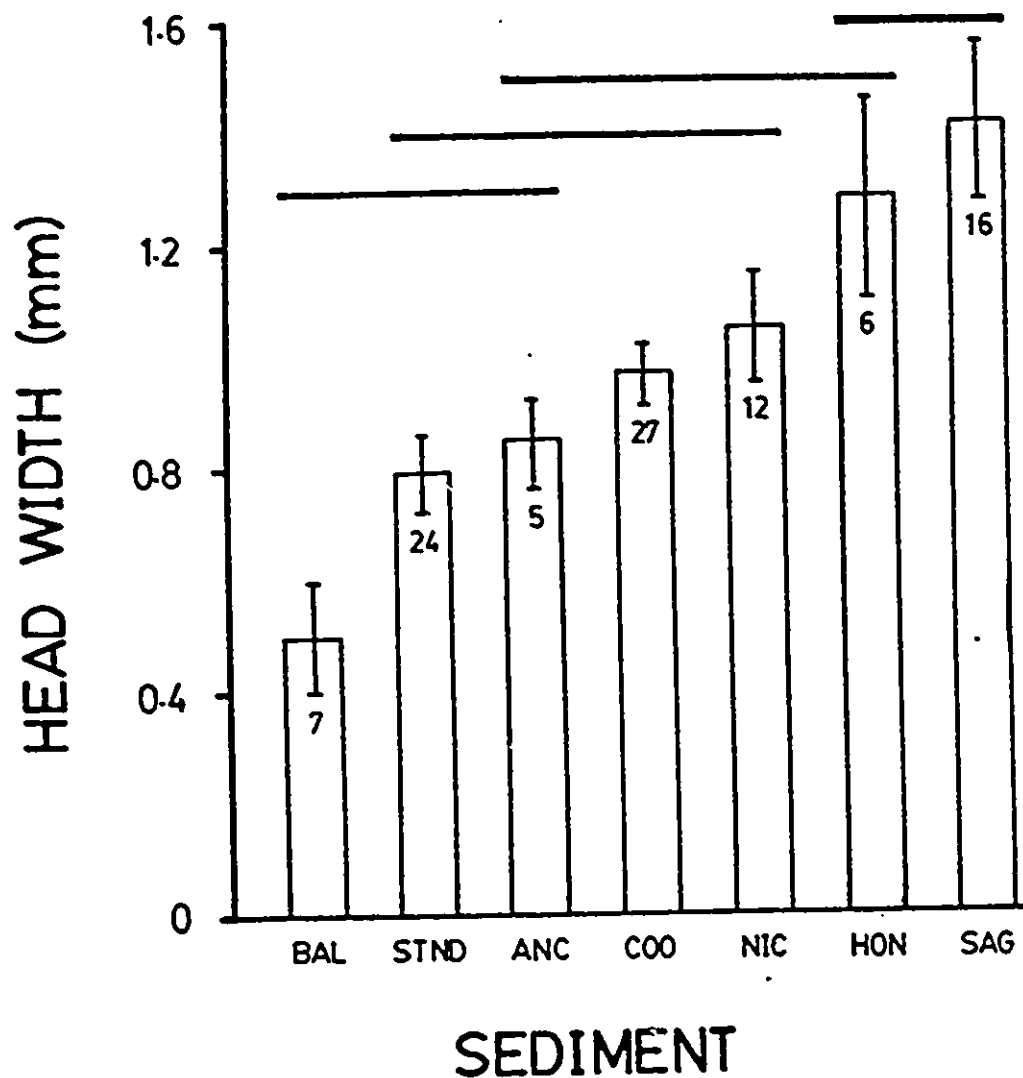


Fig. 2.3. Mean (± 1 S.E.) larval head width after 90 d growth on different sediments. Numbers associated with error bars indicate number of larvae used in analysis. The solid lines indicate sediment types that support larvae of similar size. Abbreviations for the sediment types as in Fig. 2.1.

Table 2.5. Summary of analysis of variance of larval survival among the seven different sediment types after 90 d growth.

| SOURCE OF VARIATION | df | MS | SS | F | P |
|---------------------|----|---------|--------|-------|--------|
| AMONG | 6 | 14795.6 | 2465.9 | 4.363 | < 0.01 |
| WITHIN | 19 | 10738.0 | 565.2 | | |
| TOTAL | 25 | 25533.6 | 23.8 | | |

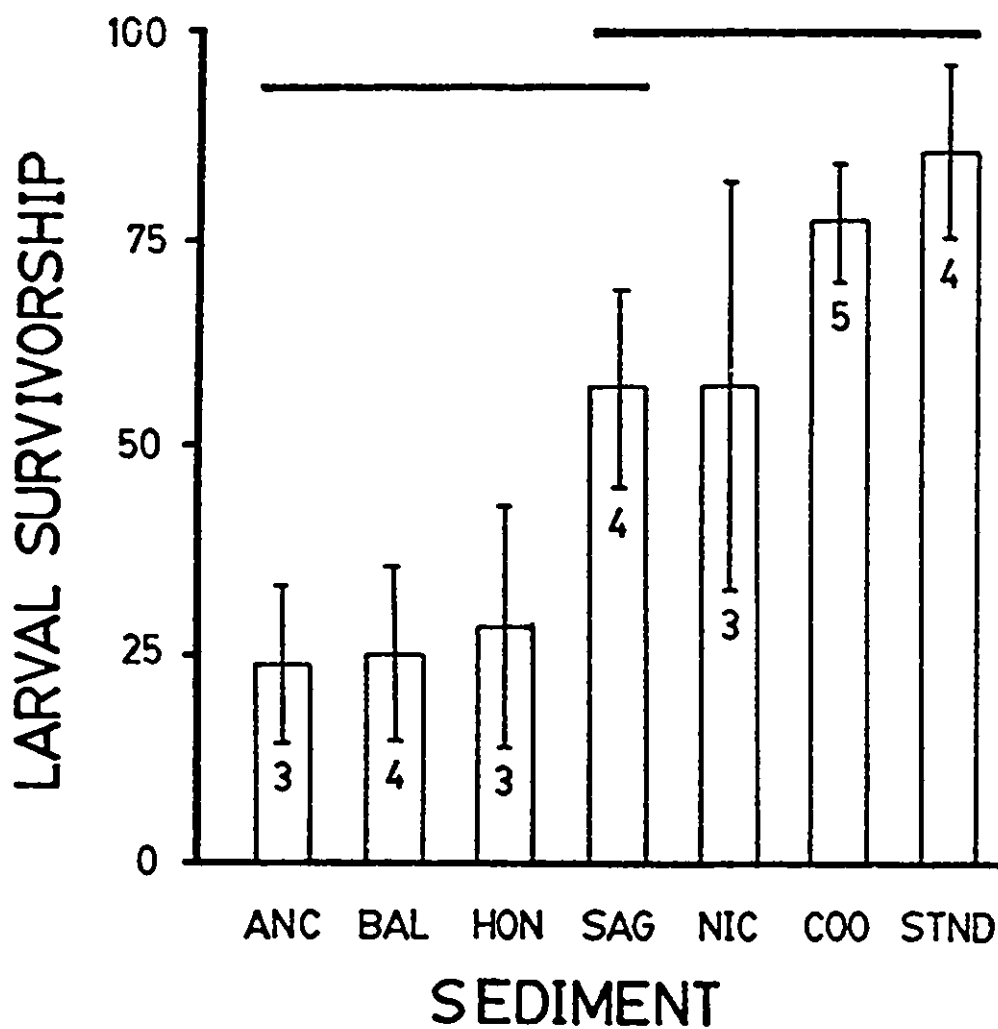


Fig. 2.4. Mean (± 1 S.E.) larval survivorship (per cent) after 90 d growth on different sediments. Numbers associated with error bars indicate the number of rearing tanks used in the analysis. Sediment types that do not differ significantly are overlain by a solid bar (SNK test, $P > 0.05$). Abbreviations for the sediment types as in Fig. 2.1.

Table 2.6. Analysis of variance of number of larvae occurring within the seven different sediment types.

| SOURCE | df | MS | SS | F | P |
|-------------|----|-------|------|-------|---------|
| SEDIMENT | 6 | 346.6 | 57.8 | 10.14 | < 0.001 |
| REPLICATE | 1 | 0.8 | 0.8 | 0.15 | ns |
| INTERACTION | 6 | 107.5 | 17.9 | 3.15 | < 0.01 |
| ERROR | 84 | 478.6 | 12.6 | | |

Note: ns, not significant at $P = 0.05$.

trials ($P > 0.05$), there was a significant interaction effect between sediment type and trials.

To determine which sediment type was harbouring the greatest number of larvae at the end of the experiment, data from the two trials were pooled and a SNK test was performed (Fig. 2.5). Results revealed that the STND-1 sediment had the fewest larvae (3.71 ± 0.667 larvae $\cdot 64 \text{ cm}^2$ cell; mean ± 1 S.E.). NIC and BAL sediment had more larvae per cell (6.57 ± 0.746 and $7.5 \pm 0.769 \cdot \text{cell}^{-1}$ respectively) than the STND-1 sediment and these two sediment types did not differ ($P > 0.05$). The four remaining sediment types (SAG, ANC, COO and HON) contained the highest number of larvae $\cdot \text{cell}^{-1}$ and they did not significantly differ from each other (Fig. 2.5; $P > 0.05$).

The presence of either an edge effect or a row/column effect could potentially account for the significant interaction observed between sediment type and replicate trial. However, for both trials, the distribution of larvae was independent of location within the tank after one week (Latin Square ANOVA, $P > 0.05$, Fig. 2.6).

The mean HW (± 1 S.E.) for all larvae in the first trial was 1.51 ± 0.011 mm; body length was 12.77 ± 0.256 mm ($n = 382$). Ninety per cent of larvae were H. limbata and 10% were H. rigida.

There was no significant difference in size of larvae among the sediment types for the first trial (ANOVA, $P > 0.05$).

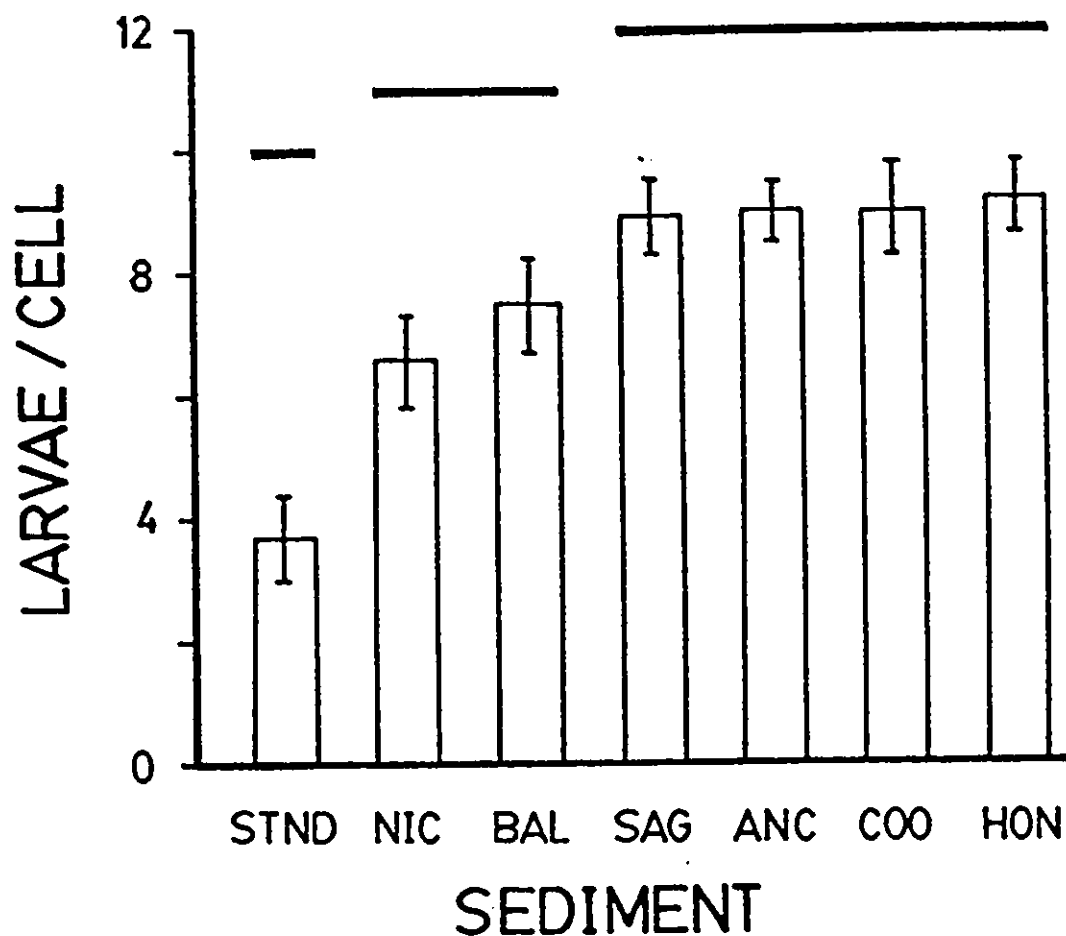


Fig. 2.5. The mean number (± 1 S.E.) of larvae located in each sediment type after 1 week. Replicates were pooled for the analysis ($n=14$ cells). Sediment types that do not differ significantly are overlain by a solid bar (SNK test, $P > 0.05$). Abbreviations for the sediment types as in Fig. 2.1.

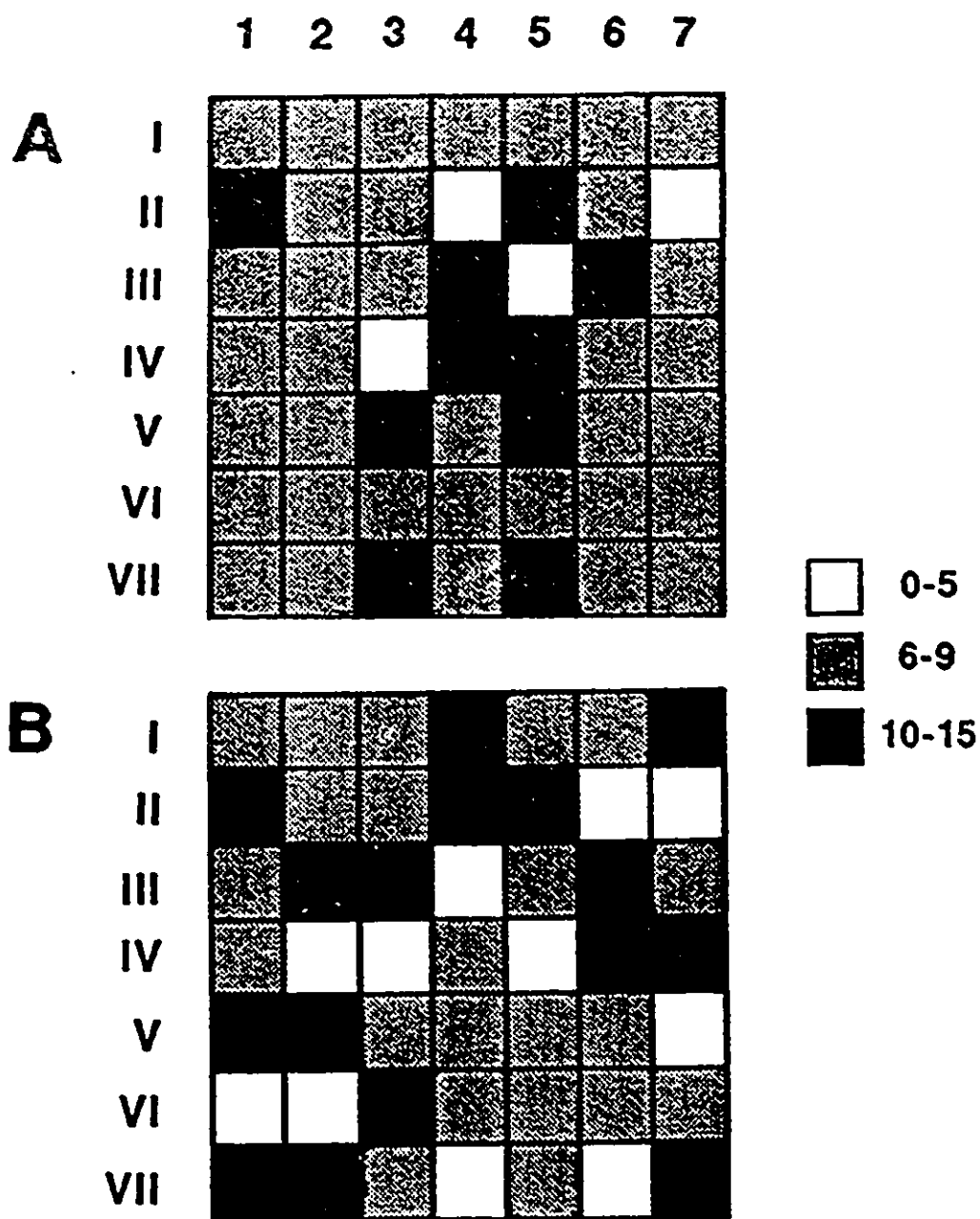


Fig. 2.6. The number of larvae occurring within each cell for trial one (top) and two (bottom) of the sediment preference experiment. The shading refers to the number of larvae found within each cell at the end of one week.

II. Sediment and Food Protocol Development

Although the STND-1 sediment did support larvae that had high survivorship, larvae reared on STND-1 grew very slowly. Also, when given a choice, fewer larvae selected STND-1 sediment than the other sediments provided (Chapter 2, part I C). As a result, a new standard sediment (STND-2) was developed to mimic Saginaw Bay sediment. Larvae reared on Saginaw Bay sediment had both excellent survivorship and growth (Chapter 2, part I B). To mimic Saginaw Bay sediment, the organic content of STND-1 sediment had to be reduced (from 28% to 9%). Accordingly, to replace much of the soil component, diatomaceous earth was selected since it is fine, of aquatic origin, and completely inorganic.

The STND-2 sediment was prepared from potter's clay, diatomaceous earth and potting soil (42, 42 and 16 percent dry mass, respectively) using procedures previously described for STND-1 sediment (Chapter 1, Part II D). The diatomaceous earth was premoistened prior to mixing to minimize aerial suspension of particles during preparation. Mean (± 1 SE) organic content of STND-2 was 8.89 ± 0.197 percent ($n = 6$).

An experiment was initiated in August 1990 to compare growth and survival among Saginaw Bay, STND-1 and STND-2 sediments. Ten replicate 1-L plastic rearing containers were each filled with 250 mL of one of the sediment types and inoculated with 7 newly hatched Hexagenia larvae. The containers were harvested after 120 d in mid-December. There

was almost complete mortality among all treatments and replicates; consequently, results will not be presented.

Although there was high mortality among all treatments, there was some concern regarding the STND-2 sediment due to the diatomaceous earth component since diatomaceous earth is commonly used as an insecticide for terrestrial organisms. Therefore, a new synthetic sediment was developed using fine silica sand to replace the diatomaceous earth component (R.J. Thibert, University of Windsor, personal communication). The particle size distribution of this new sediment (STND-3) also mimicked the particle size distribution of Saginaw Bay sediment. The STND-3 consisted of fine sand:clay:soil in a 42:42:16 ratio (dry wt; Table 2.7) and is mixed using procedures previously described for STND-1 sediment. The sand portion was presieved and mixed such that two-thirds consisted of particles 90-180 μm in diameter and one third was 180-250 μm .

The clay portion of the STND-3 consisted of Lewiscraft^R sculptor's clay (Lewiscraft Ltd., Toronto, ON M1S 2S2) in a powdered form (instead of the premoistened potter's clay previously used for all standard sediments). Dry sculptor's clay contains very few contaminants (specifically, PCBs) compared to premoistened potter's clay (Z.E. Kovats, International Joint Commission, Windsor, ON, personal communication), possibly due to the manufacturing company adding oil as a wetting agent. After 30 June 1991, all standard sediments that required clay as a component were

Table 2.7. Ingredients used to produce 1L of STND-3 sediment. All masses assume 0 moisture content.

| CONSTITUENT | DRY MASS (g) |
|--|--------------|
| Sulptor's Clay | 285 |
| Fine Sand (90 - 180 μm) | 190 |
| Coarse Sand (180 - 250 μm) | 95 |
| Potting Soil | 210 |

mixed with the sculptor's clay instead of potter's clay.

The experiment designed to compare larval growth between the STND-1 and STND-2 sediments was repeated with some modification, including inclusion of the STND-1 sediment (see sediment and feeding protocol experiment below).

Methods

In addition to comparing growth of larvae among the three standard sediments (STND-1, STND-2 and STND-3) and Saginaw Bay sediment, I also wanted to compare the influence of two different food types on larval growth. Because of difficulties I had had in obtaining alfalfa powder, I wanted to compare the standard food mixture with the standard food mixture minus alfalfa. The experimental design was a 4 (sediment type) x 2 (food type) factorial design with 8 replicates per treatment (Table 2.8). The four sediment types were Saginaw Bay sediment (SAG; collected in September 1989 (Chapter 2, Part I A)), STND-1, STND-2, and STND-3. All standard sediments used in this experiment were mixed with the sculptor's clay in lieu of the potter's clay. The two food treatments were 1) the standard food mixture consisting of Tetramin^R, yeast and alfalfa and 2) the standard food mixture minus alfalfa (i.e., Tetramin^R and yeast). The total amount of food provided was identical in all treatments.

A random number table was used to arrange the 64 plastic rearing tanks (1 L) required for the experiment on the laboratory counter. Two hundred mL of each sediment type was

Table 2.8. Experimental design for sediment and food experiment. Numbers indicate the number of replicate tanks each stocked with 8 larvae.

| FOOD TYPE | DURATION | SEDIMENT TYPE | | | |
|--------------------|----------|---------------|--------|--------|--------|
| | | SAG | STND-1 | STND-2 | STND-3 |
| WITH ALFALFA | 30d | 3 | 3 | 3 | 3 |
| | 75d | 5 | 5 | 5 | 5 |
| WITHOUT ALFALFA | 30d | 3 | 3 | 3 | 3 |
| | 75d | 5 | 5 | 5 | 5 |

added to each of 8 replicate containers. Distilled water was added to all tanks; tanks were continuously aerated with filtered air. Colour codes on the lip of the rearing tanks were used to identify the food treatment to be administered. One week prior to larval addition, 3 mL of concentrated food (Chapter 1, Part II C), consistent with the experiment's food treatment, was added to each tank.

Eight larvae per tank were added on 30 June 1991 hatched from eggs collected 13 June 1991. Three replicates of each treatment were harvested after 30 d; the remaining 5 replicates per treatment were harvested after 75 d. Head width and body length of all larvae were measured, and larval survivorship for each tank was determined.

Results

There was complete mortality in the Saginaw Bay sediment treatments, and thus, this treatment was excluded from the analysis. The following results pertain to larval size and survivorship among STND-1, STND-2 and STND-3 sediments only.

30 D

Results of a two-way ANOVA indicated that after 30 d, neither sediment type nor food type significantly influenced larval size (Table 2.9). Larval head width ($\bar{X} \pm \text{S.E.}$) ranged from 0.29 mm \pm 0.006 (STND-2 sediment with the no alfalfa food) to 0.42 mm \pm 0.030 (STND-3 sediment with the no alfalfa food) (Fig. 2.7a).

Table 2.9. Summary of two-way ANOVA of the influence of sediment, food and their interaction on larval size (head width) after 30 d.

| Source of Variation | Degrees of freedom | Sum of Squares | F | p |
|---------------------|--------------------|----------------|-------|------|
| Sediment | 2 | 0.074 | 3.110 | n.s. |
| Food | 1 | 0.000 | 0.009 | n.s. |
| Interaction | 2 | 0.058 | 2.443 | n.s. |
| Error | 12 | 0.142 | | |

Note: ns, not significant at $P = 0.05$.

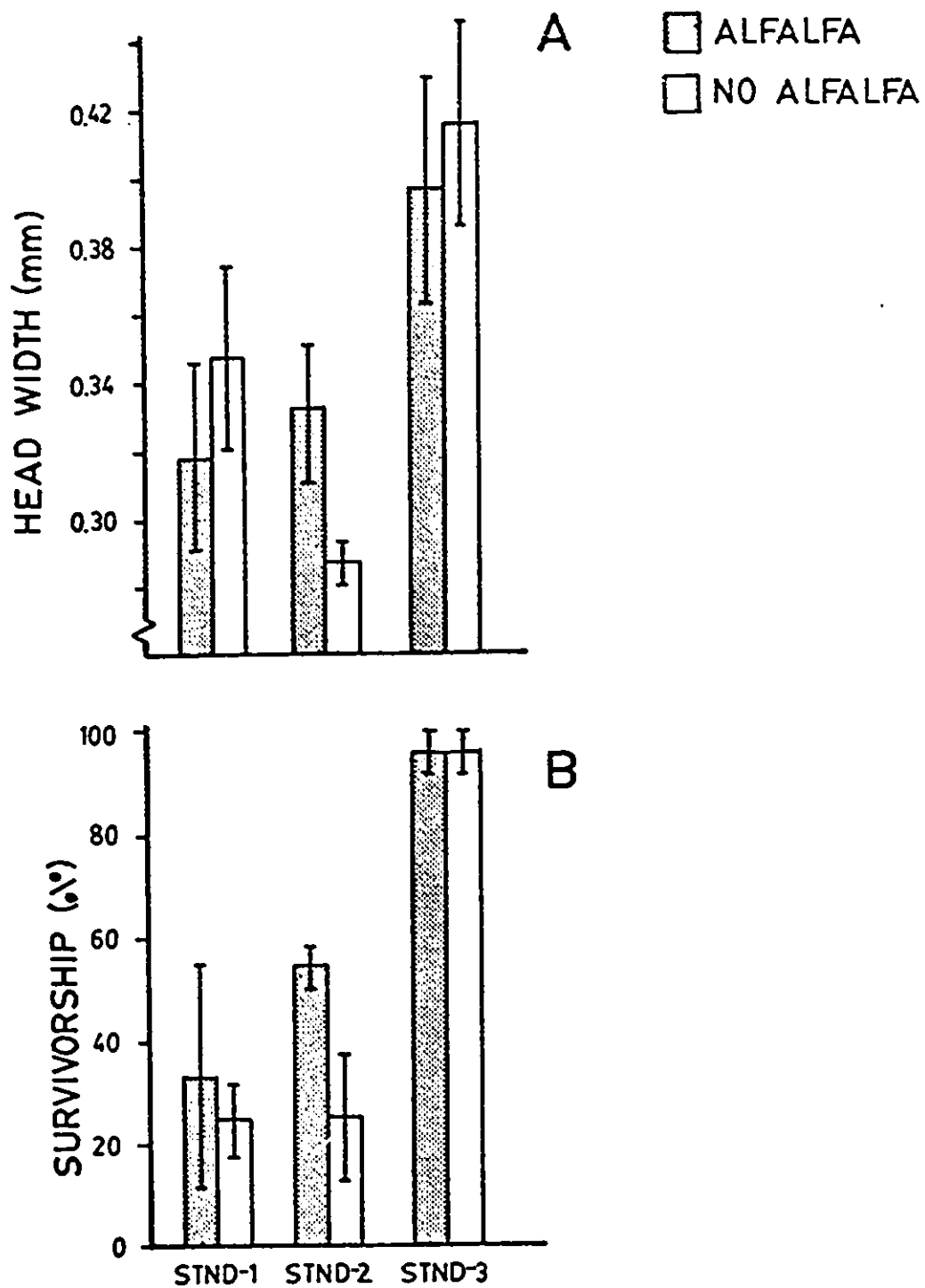


Fig. 2.7. Mean (± 1 S.E., $n=3$) (A) larval head width (mm) and (B) larval survivorship (per cent) after 30 d growth on three different standard sediments and two different food types. Shaded histograms indicate food with alfalfa; open histograms indicate food without alfalfa.

After 30 d, larval survivorship was significantly influenced by sediment type ($P < 0.001$), but not by food nor the interaction of substrate and food (Table 2.10). Larvae reared in STND-3 sediment exhibited higher survivorship (95.8 % for both food types) than larvae reared on either STND-1 (30-35 %) or STND-2 (25-55 %) sediment types (Fig. 2.7b).

75 D

After 75 d, neither sediment, food type, nor the interaction of the two factors influenced the size of larvae ($P > 0.05$, Table 2.11). However, larvae reared on STND-2 sediment did tend to be smaller than larvae reared on either of the other two sediment types (Fig. 2.8a).

Results of a two-way ANOVA indicated that after 75 d growth, sediment type did influence survivorship of larvae ($P < 0.005$, Table 2.12). Larvae reared in STND-3 sediment had higher survivorship than larvae reared in either STND-1 or STND-2 sediment (Fig. 2.8b). Neither food type nor the interaction of sediment and food significantly influenced larval survivorship ($P > 0.05$, Table 2.12).

Table 2.10. Summary of two-way ANOVA of the influence of sediment, food and their interaction on larval survivorship after 30 d.

| Source of Variation | Degrees of freedom | Sum of Squares | F | p |
|---------------------|--------------------|----------------|--------|--------|
| Sediment | 2 | 1.543 | 20.679 | <0.001 |
| Food | 1 | 0.070 | 1.884 | n.s. |
| Interaction | 2 | 0.068 | 0.907 | n.s. |
| Error | 12 | 0.448 | | |

Note: ns, not significant at $P = 0.05$.

Table 2.11. Summary of two-way ANOVA of the influence of sediment, food and their interaction on larval size (head width) after 75 d.

| Source of Variation | Degrees of freedom | Sum of Squares | F | p |
|---------------------|--------------------|----------------|-------|------|
| Sediment | 2 | 1.694 | 2.294 | n.s. |
| Food | 1 | 0.002 | 0.008 | n.s. |
| Interaction | 2 | 1.809 | 2.451 | n.s. |
| Error | 24 | 8.859 | | |

Note: ns, not significant at $P = 0.05$.

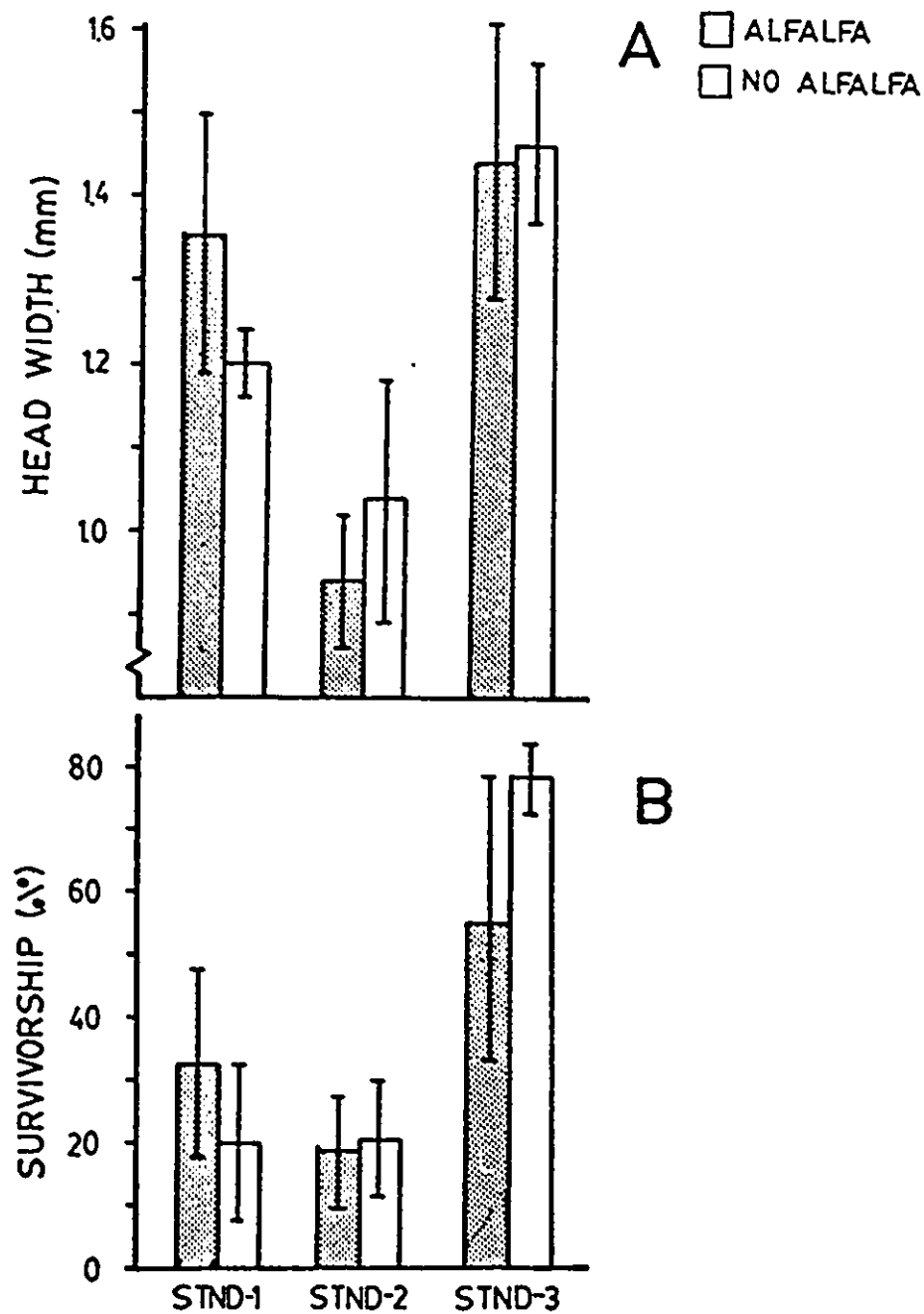


Fig. 2.8. Mean (± 1 S.E., $n=5$) (A) larval head width (mm) and (B) larval survivorship (per cent) after 75 d growth on three different standard sediments and two different food types. Shaded histograms indicate food with alfalfa; open histograms indicate food without alfalfa.

Table 2.12. Summary of two-way ANOVA of the influence of sediment, food and their interaction on larval survivorship after 75 d.

| Source of Variation | Degrees of freedom | Sum of Squares | F | p |
|---------------------|--------------------|----------------|-------|--------|
| Sediment | 2 | 1.202 | 6.898 | <0.005 |
| Food | 1 | 0.000 | 0.001 | n.s. |
| Interaction | 2 | 0.297 | 1.705 | n.s. |
| Error | 24 | 2.090 | | |

Note: ns, not significant at $P = 0.05$.

III. Mass Rearing

Methods

I investigated mass rearing techniques using three large aquaria (160 L, ca. 2700 cm²). STND-1 sediment was added to each aquarium to a depth of 4 cm (ca. 11 L); the aquaria were filled with aerated, distilled water. Water was continuously aerated using a vacuum-pressure pump (Barnant Co. model 400-1901); air was filtered through both a carbon filter and a filter tube (Balston DFU Grade BK). To ensure proper aeration, throughout the experiment, water also was circulated within the tanks using a water pump (AquaClear^R 200). Two weeks prior to adding larvae, an excess of food (9 g of ground Tetramin^R) was mixed into the sediment in each aquarium (3.33 mg/cm²). Netting covered the aquaria to trap any emerging subimagoes.

Larvae were hatched from eggs collected 21 July 1990 and added to aquaria on 15 February 1991 (600 per aquarium). Larvae were fed 4.5 g ground Tetramin^R flakes twice weekly (1.11 mg/cm²). This amount of food is consistent with the amount of food administered to larvae (per capita) in the sediment/food protocol experiment (see above).

Conductivity, pH and dissolved oxygen were determined for each aquarium throughout the experiment. One-half of the water was removed from the tanks and replaced every 60 d (Friesen 1981).

On 17 May 1991 (=60 d growth), subimagoes began to emerge from the aquaria. Imagoes were retrieved from netting that

covered the rearing tanks and preserved in 70% ethanol. However, since the netting did not isolate each tank, it could not be determined from which tanks the mayflies emerged. Body length for all preserved adults was measured with calipers to the nearest 0.1 mm; male imagoes were identified to species.

Rearing tanks were harvested 24 June - 18 July 1991 (126-147 d). Sediment was scooped out of the tanks with a fish net (mesh size 1 mm) and sieved through a 1-mm sieve. Larvae that were smaller than 12 mm or larger than 20 mm (including black wing pad larvae) were preserved in 70% EtOH. Larvae that were between 12 and 20 mm in BL were removed and either given to the Great Lakes Institute (University of Windsor) for a bioassay study or to Dr. Jan J.H. Ciborowski (Department of Biological Sciences, University of Windsor) for a 21-d bioassay. Larvae in the latter experiment also were used to determine the accuracy of photographs for measuring larvae.

Results

Larval survivorship in the three tanks, excluding the subimagoes, averaged 36% (Table 2.13). The subimagoes were not included in the survivorship values because I could not distinguish from which tanks the mayflies emerged since the netting covering the aquaria did not have partitions between the aquaria. Therefore, the reported survivorship values are underestimates since the adult imagoes were not included as surviving larvae. Furthermore, the number of small larvae was probably underestimated because the sieve mesh size used

Table 2.13. Number, size, and survivorship of larvae removed from the mass rearing tanks after approximately three months.

| Tank | # of larvae | | | Survivorship (%) |
|------|-------------|----------|--------|---------------------|
| | <12 mm | 12-20 mm | >20 mm | |
| A | 16 | 140 | 36 | 32 |
| B | 0 | 245 | 25 | 45 |
| C | 2 | 440 | 31 | 31 |

during harvesting was large and the sediment was only superficially examined for small larvae.

One hundred and twenty-two subimagoes emerged between 17 May and 18 July 1991; 49 females (mean body length \pm S.E. = 19.30 ± 0.388 mm) and 73 males (16.71 ± 2.480 mm). All male imagoes were H. limbata.

IV. Photographic Estimates of Larval Size

Methods

I wanted to determine if photographs of larvae taken before and after an experiment could provide an accurate estimate of larval size. To accomplish this, I used larvae from a short-term (21 d) bioassay experiment (Ciborowski et al. 1991)

Prior to adding 10 "half-grown" larvae (BL = 9-15 mm) to each of 25 rearing tanks, 5 larvae were placed in each of 50 petri dishes to be photographed. I considered five larvae per dish to be a manageable number so that larvae could be accurately counted and photographed. Two petri dishes containing larvae (total = 10 larvae) were designated for each experimental tank. Labels on the bottom of each petri dish identified the tank for which larvae were designated. I marked several 1-cm scale lines using indelible ink on the bottom of the petri dishes. The identifying labels and the 1-cm scale lines were used in the photographs to indicate which replicate tank larvae were assigned and to calibrate the photograph.

To take the photographs, I used a 35 mm camera loaded with ASA 100 Kodacolor^R colour print film with a shutter speed of 1/125 s. The camera was secured to a camera mount with two tungston flood lights illuminating the petri dishes.

Larvae in the petri dishes were photographed before they were added to the experimental tanks. Twenty-one days later,

at the conclusion of the experiment, larvae were removed from the tanks and placed into the original petri dishes that corresponded with each experimental tank replicate. Larvae were photographed and preserved in 70% ethanol.

To determine if photographs of larvae provide an accurate representation of larval size, both the preserved larvae obtained at the end of the 21-d experiment, and their photographs were measured to the nearest 0.075 mm using a dissecting microscope fitted with an ocular micrometer. Four scale marks located on the bottom of each petri dish were measured both in the photograph and on the 'real' petri dish for calibration. The measurements of the photographed larvae were multiplied by this calibration factor to provide an estimate of the true larval size. These estimated measurements were compared to the actual larval measurements. The two measurement types (actual vs. estimated) were compared for both HW and BL using a single linear regression. If the relationship between the actual and estimated values was perfect, the slope of these regression lines would be equal to one. Therefore the relationship between the two measurements was tested against a null hypothesis of slope equal to one.

Results

The actual HW and BL measured from preserved larvae were regressed with the HW and BL estimates obtained from larval photographs at the conclusion of the 21-d bioassay. The

equation of the line for estimated (EST) HW vs actual (ACT) HW was

$$\text{ACT HW} = 0.7608 \text{ EST HW} + 0.3819 \quad (1)$$

(S.E. of slope = 0.088). The equation of the line for estimated BL vs actual BL was

$$\text{ACT BL} = 0.9019 \text{ EST BL} + 1.5939 \quad (2)$$

(S.E. of slope = 0.9410). The correlation coefficient for the regression between both the actual HW and BL and estimated HW and BL was 0.84 (Figs. 2.8, 2.9).

The regression line for the estimated HW vs. actual HW was marginally significantly different from 1.0 ($P < 0.05$; Fig. 2.9), indicating that there was a significant difference between actual and estimated HW measurements. However, the slope of the regression for the BL estimate vs. actual did not significantly differ from one ($P > 0.05$; Fig. 2.10) suggesting that there is no significant difference between actual BL measurements and estimated BL measurements.

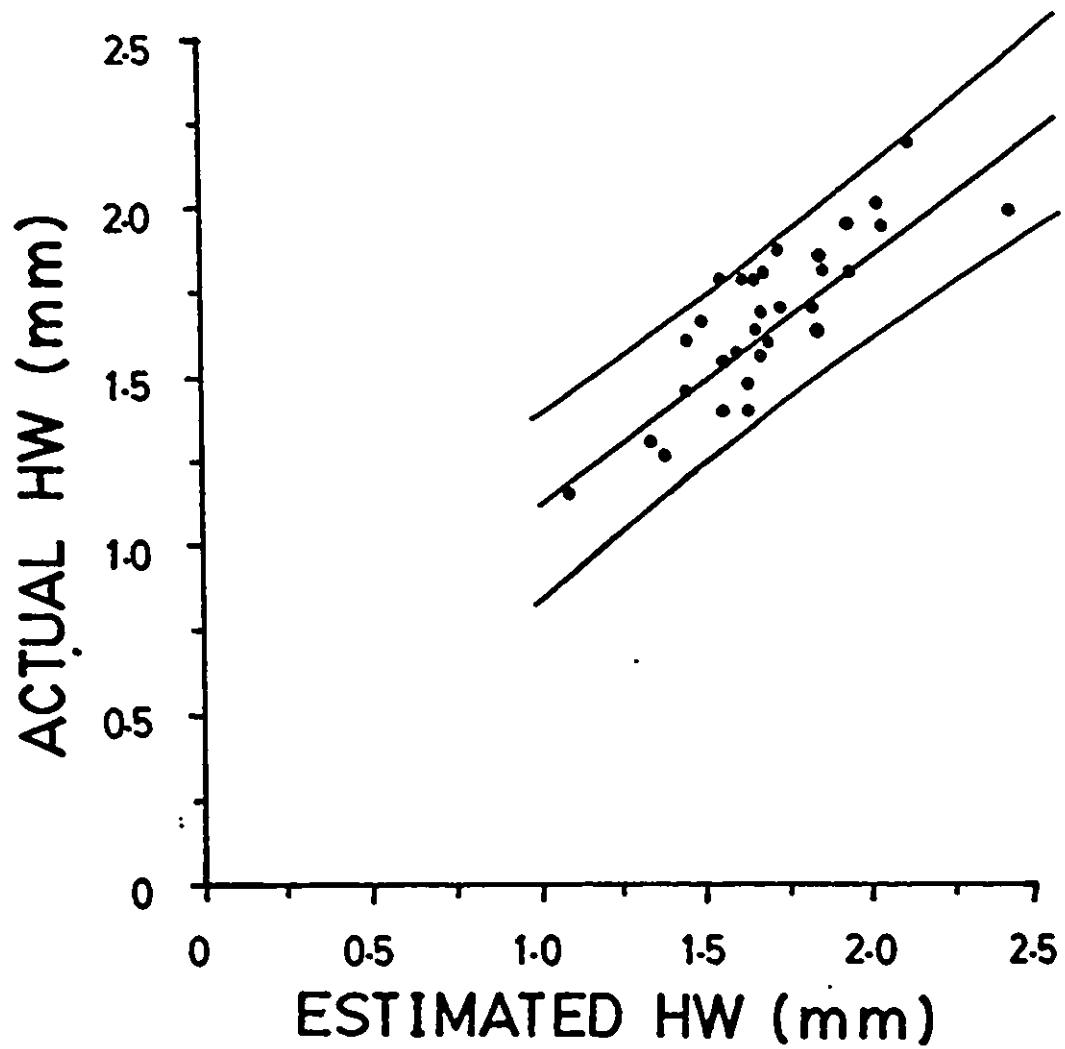


Fig. 2.9. Relationship between estimated larval head width and actual HW. Regression line takes the form: [estimated HW] = $0.382 + 0.761$ [actual HW]. The slope of the line significantly differs from 1.0 ($P < 0.05$).

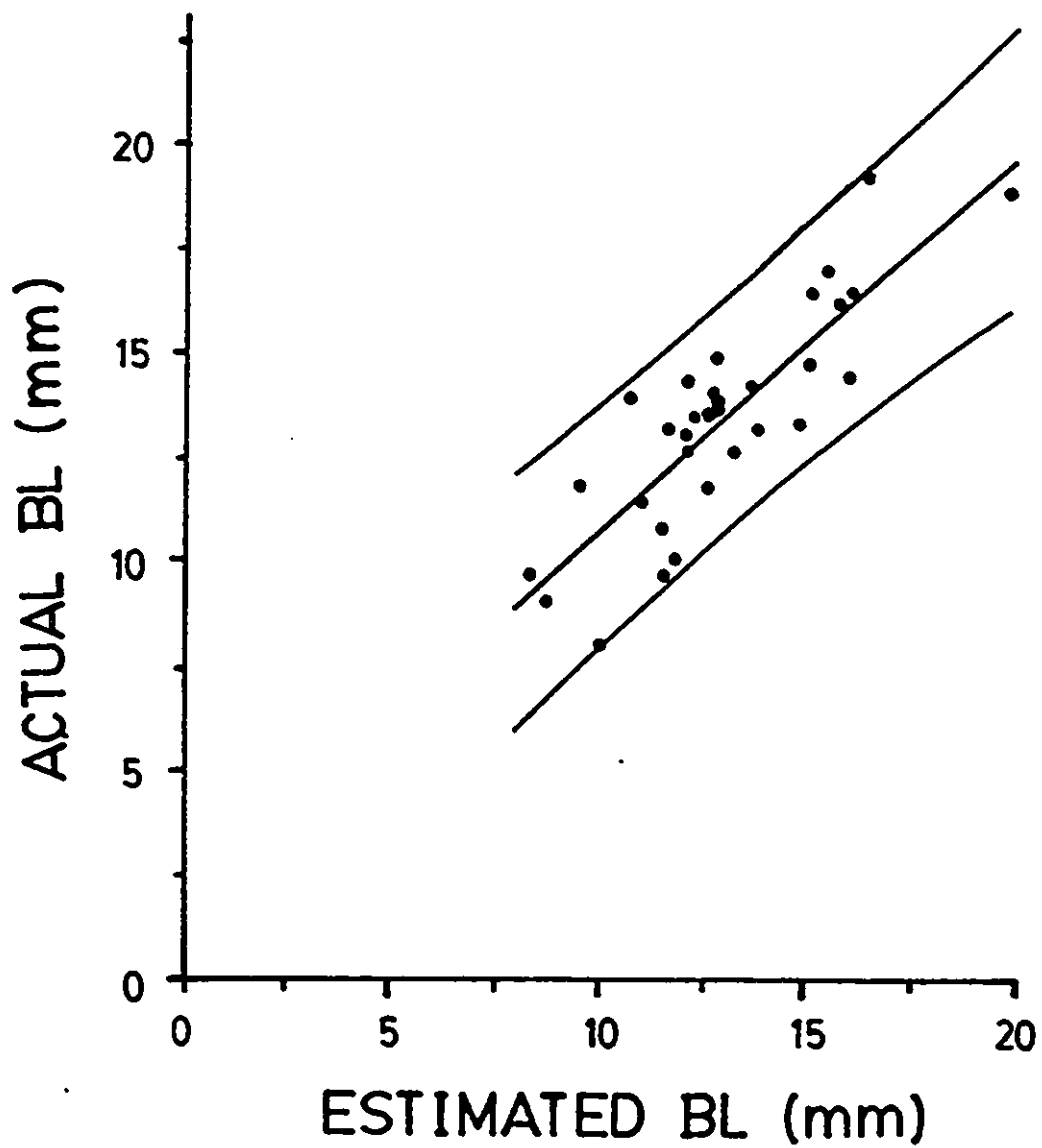


Fig. 2.10. Relationship between estimated larval body length and actual BL. Regression line takes the form: [estimated BL = 1.594 + 0.902 [actual BL]]. The slope of the line does not significantly differ from 1.0 ($P > 0.05$).

DISCUSSION

Sediment and Food Protocol Development

The coupled laboratory experiments (larval size/survivorship and sediment selection by larvae) were used to determine which natural sediment best supports larvae that grow quickly while maintaining high survivorship. A standard sediment that mimics the particle size distribution and organic content of the natural sediment found most suitable for larval growth was developed based upon the results of these studies. The natural sediments used in these experiments were collected from sites that currently support or had historically supported large Hexagenia populations. The STND-1 sediment was included in the study since this sediment had previously been used for successful laboratory rearing of Hexagenia (Corkum and Hanes 1992; Hanes and Ciborowski 1992; Droulliard et al. Ms. in prep.).

Typically, substrate selection experiments are designed to investigate the distribution of organisms among substrates within a single waterbody (see Corkum et al. 1977; Wright and Mattice 1981b). Often, some sediments included in selection experiments are substrates that the study organism does not normally colonize. Because I wanted to develop a standard sediment based upon characteristics of a sediment known to support Hexagenia populations, I presented larvae with sediments collected from several different waterbodies. I use the term selection only to refer to the presence of larvae within sediment. The sediment selection experiment was not

designed to distinguish between a larva's preference for a desirable sediment and avoidance of a poor sediment.

The larval growth experiment revealed that Saginaw Bay sediment was the only sediment type in which larvae both grew quickly and yet maintained high survivorship. The sediment selection experiment revealed that there were four natural sediments (SAG, ANC, COO, HON) that larvae occupied most frequently. Therefore, of the sediments examined, Saginaw Bay sediment was the only sediment type that provided larvae with good survivorship, large size and when given a choice, larvae selected that sediment type.

Hexagenia larvae require soft, silty sediment for production and maintenance of burrows (Hunt 1953; Eriksen 1964; Rasmussen 1988). If the sediment in which larvae burrow does not maintain a burrow effectively, then the time that larvae must devote to maintaining burrows (and not feeding) may impede larval growth (Hanes and Ciborowski 1992). Additionally, sediment texture may influence the rate of food intake (Zimmerman and Wissing 1978) and oxygen consumption (Eriksen 1963b, 1964). Wright and Mattice (1981b) found that larvae selected sediment types characterized as adhesive mud and fine sandy clay. Saginaw Bay sediment consists of equal portions of fine sand and silt. However, the particle size distribution of Saginaw Bay sediment was similar to that of the sediments collected from Balsam Lake, Cook's Bay and Honey Harbour, so it is unlikely this is the only factor responsible for the observed differences in size, survivorship and substrate selection by larvae.

Organic content may also contribute to larval growth, since amount of organic material is typically considered indicative of food available (Zimmerman and Wissing 1978). Therefore, one may anticipate a positive correlation between organic matter and larval growth. However, of the natural sediments examined, Saginaw Bay had only an intermediate amount of organic matter (8 per cent in a range of 2-18 per cent). If some of the organic matter is inedible (e.g., lignin and cellulose), then the total amount of organic matter will not accurately reflect the available food. Additionally, since food was provided to larvae within all tanks and treatments, variation in larval size and survivorship attributed to differences in organic content among the sediment types should be minimized.

In the substrate selection study, the sediments that contained the most larvae (SAG, ANC, COO, HON) had intermediate values of organic content. Sediments that harboured the fewest larvae had the least (BAL) and most (NIC, STND-1) organic matter. Although Wright and Mattice (1981b) did not find a significant correlation between organic content and number of larvae in a sediment type, they did detect a tendency for larvae to select sediment that had higher per cent organic content. The ease with which larvae penetrated a substrate was correlated with organic matter and the ease of penetrability by a larvae was determined to be a primary factor for selecting substrate type (Wright and Mattice 1981b). Since organic matter content and penetrability by mayflies are associated, organic matter may influence burrow

production and maintenance.

Perhaps low organic content in sediment is undesirable because it indicates low availability of food, whereas very high organic content also may be undesirable because the organic material reduces cohesion of sediment, inhibiting burrow production. In a Michigan lake, Hexagenia larvae were absent from sediment with high organic content, but larvae were abundant in sediments that had a similar particle size distribution, but lower organic content (Eriksen 1966). This anecdotal evidence supports the results of the coupled sediment experiments indicating that an intermediate level of organic content is best for supporting mayfly populations.

Although rearing larvae on the STND-1 sediment results in high larval survivorship, the larvae are comparatively small. The extremely high organic content (28 %) of this sediment may interfere with burrow production. Additionally, while the per cent organic content is high, if the organic matter is inedible, then the larvae may ingest large amounts of sediment, but little food content (Zimmerman and Wissing 1978).

Based upon the results of the coupled laboratory experiments, two standard sediments (STND-2, STND-3) were developed to provide texture and organic content that more closely matched Saginaw Bay sediment attributes than the previously developed standard sediment. It is likely the combination of the particle size distribution and the organic content of Saginaw Bay sediment that resulted in the best cohesive sediment for burrow production.

The experiment comparing growth of larvae among the STND-1, -2, -3 and Saginaw Bay sediments indicated that while larval size was not significantly influenced by sediment type, larval survivorship was. Larval survivorship was highest in the STND-3 sediment. Unfortunately, I could not compare the larval size and survivorship between the STND-3 sediment and Saginaw Bay sediment due to complete mortality in the natural sediment. Possibly, the prolonged storage of Saginaw Bay sediment made that substrate unsuitable for larval growth.

I recommend the STND-3 sediment as the most appropriate standard sediment for laboratory rearing of Hexagenia larvae. Because larval growth is so variable, a standard sediment, would aid in quality assurance and control of experiments conducted in different laboratories. Also, since the contaminant burden of the STND-3 sediment is minimal (Ciborowski et al. 1991), it is an excellent reference sediment for bioassay or sediment dilution series studies (see Ciborowski et al. 1991).

Results of the study that compared two different food types indicated that there was no difference in larval size and survivorship between the two food types. Larvae grew equally well when fed either Tetramin^R, yeast, alfalfa mixture or Tetramin^R, yeast only. Hanes and Ciborowski (1992) found that although food quantity did influence larval growth, the correlation was weak. Most authors report stronger correlations between food quantity and size of aquatic invertebrates (Brachycen'rid caddisflies: Gallepp 1977, larval black flies: Colbo and Porter 1979) than between food quality

and size (mayflies: Cianciara 1979, Webb and Merritt 1987, Söderström 1988, Rosillon 1988).

The original food mixture (Tetramin^R, yeast and alfalfa) was a recommended food source for rearing a Missouri population of Hexagenia larvae in the laboratory (M.G. Henry, Minnesota Cooperative Fish and Wildlife Research Unit, St. Paul, MN, personal communication). However, it can be difficult to obtain alfalfa powder from local retail outlets. Since my larvae grow equally well on both food sources, either food type could be used for successful rearing of Hexagenia larvae.

The size/survivorship variation observed in the sediment: larval growth experiment underlines the amount of variability (at a minimum) that can be expected among results of different laboratories that each use their own materials for experiments. Standardization of sediment required for rearing Hexagenia would facilitate comparison of results both within and between laboratories. I have determined that the STND-3 sediment provides excellent growth of mayfly larvae. Additionally, a standard mayfly food consisting of either Tetramin^R, yeast and alfalfa or Tetramin^R and yeast only work equally well at stimulating mayfly growth.

Mass Rearing

The mass rearing experiment was successful since large numbers of mayflies were reared from first instar larvae to late instar larvae within three months. In natural populations, Hexagenia mayflies require one to four years to

reach maturity (Hunt 1953, Heise *et al.* 1987, Giberson and Rosenberg 1992). Hunt (1953) reared larvae to maturity on field-collected sediment in 8 to 12 months. Although Fremling (1967) reportedly reared larvae to maturity on garden soil and composted hay in three months, using the same procedure, Thomforde and Fremling (1968) required 6 to 9 months before they observed sporadic emergence of laboratory-reared subimagos. Large numbers of mayflies did not emerge until after 10 to 13 months' growth.

The survivorship of the mayflies in the mass rearing experiment was at least 30 to 40 per cent. Although these values are underestimates, this survivorship is higher than reported for other mass rearing procedures. Fremling (1967) and Thomforde and Fremling (1968) reported low survivorship with their mass rearing procedures (<7 %).

It is noteworthy that in many laboratory experiments where Hexagenia larvae are reared in small containers, larvae survive in the laboratory for much longer duration without reaching maturity (see Wright *et al.* 1982; Corkum and Hanes 1992; Hanes and Ciborowski 1992; Giberson and Rosenberg 1992). Perhaps space is limiting in the small rearing tanks. If larvae cannot properly maintain a burrow due to insufficient space, feeding, which usually is continuous (Zimmerman and Wissing 1978), may be interrupted and larval growth impeded. Information relating larval growth in small containers at equivalent to larval growth in large containers (more similar to field conditions) would be valuable. This experiment is currently in progress (J.J.H. Ciborowski, E.C. Hanes and L.D.

Corkum, University of Windsor, personal communication).

Despite the biased recovery of large larvae due to the large sieve size used when sampling, the size variation in larvae appeared to be less than that of other laboratory-reared populations (personal observation). This rearing technique is therefore recommended for mass rearing larvae of uniform size. To ensure high survivorship of larvae I recommend rearing larvae for no longer than 10 weeks to avoid larvae that are either in the last stage of development (i.e., black wing pad stage) or emerge as subimagos.

Photographic Estimates of Larval Size

I have determined that using photographs as a means to estimate larval size is a valuable alternative to measuring actual larvae. Since the slope of the relationship between actual BL and estimated BL did not differ from 1.0, BL is a more accurate estimate of larval size than HW when using photographic estimates of size. However, because the photographs for this experiment were slightly overexposed, the larval body parameters were not always easy to discern. Additionally, since living larvae are active, photographs frequently contained images of larvae that were on their side, and thus measurements were difficult to determine. These factors contributed to the relatively poor correspondence between measurements. However, since the errors are normally distributed and mean HW and BL are used as estimates of larval size (and not individual sizes) the final estimate of mean larval size should be quite accurate.

The technique of photographing larvae has subsequently been used for other experiments (Ciborowski, Hanes and Corkum, unpublished data). For these experiments, we are employing two modifications. Firstly, we are slightly underexposing the photographs, which makes the larval bodies more easy to discern. Secondly, larvae are anaesthetized with club soda prior to photographing them. Larvae that are subdued can easily be manipulated so that their dorsal side is completely visible, allowing HW and BL to be readily determined in the photograph. Both of the above modifications have improved the accuracy of the photographs and estimates of larval size (unpublished data).

To summarize, photographing larvae is a feasible alternative to obtaining size measurements of larvae when using actual larvae is not available. The photographs provide an accurate record of the number and size of larvae before they are added to experimental treatments. Possibly, photographs of live larvae may provide a more accurate record of larval size than measuring preserved larvae, since preservatives often change larval size (Britt 1953; Heise et al. 1988, personal observation).

GENERAL CONCLUSIONS AND RECOMMENDATIONS

My studies have shown that Hexagenia is a good study organism to use for examining life history characteristics since all life stages (egg, larvae, adult) are readily identified and measured.

Mean female size of Hexagenia adults decreased through the emergence period, 29 June to 5 Aug, 1990 in Lake St. Clair. Near the end of the emergence period, even if full reproductive development has not been achieved (indicated by small body size), it may be more advantageous for a female to emerge and reproduce, than to risk another year of predation. A trade-off between survival and reproductive value for female imagoes likely exists.

There was little variation in male imago size over the emergence period. Small size in male imagoes may confer greater manoeuvrability in mating swarms. Since there is no trade-off between rapid maturation and reproductive value in males, male imago size remains constant throughout the emergence period.

Eggs monitored at room temperature began to hatch approximately two weeks after female imagoes were collected and eggs removed. Complete hatching (>90%) occurred within a few days of initial hatching. Eggs that were kept in cold storage for six months also exhibited high viability when returned to room temperature. Therefore, eggs can be maintained in cold storage for extended periods of time and

still exhibit excellent survival.

I detected no evidence of an egg diapause for eggs collected from the Lake St. Clair population of Hexagenia. Although delayed hatching enforced by an egg diapause does not contribute to larval size variation in this population, delayed hatch resulting from dormancy of eggs over winter (equivalent to cold storage) may occur and as such would contribute to size variation of larval field populations.

Attempts at artificial insemination of field-collected adults met with limited success. Since no interspecific viability between H. limbata and H. rigida was detected in the laboratory, interspecific mating is probably not an important factor contributing to size variation in the field. Parthenogenesis is a minor influence on size variation in the field since less than five per cent of Hexagenia eggs hatched parthenogenically.

I found differences in size of eggs obtained from different females using visible egg area as an indication of size. The relationship between maternal size and egg size is not clear. Egg size decreased after the first week of adult emergence, concurrently with a decrease in female size. However, analysis indicated that collection date (as opposed to female size) was the overriding factor contributing to egg size.

Maternal size was not correlated with size of first instar larvae although body length of first instar larvae did vary among females. This finding is consistent with the

results of the study examining maternal size and egg size, of which egg size is most likely the best predictor of first instar size.

Maternal size was negatively correlated with size of 40-day-old larvae (approx. one-quarter of mature size). Large females produced smaller offspring than small females. Maternal size also influenced survivorship of 40-day-old larvae. Females of the mean maternal size produced larvae with the highest survivorship. Females that were either larger or smaller than the mean size, produced larvae with lower survivorship. Thus, a female of the mean maternal size may have a greater 'realized' fecundity than a larger female if a greater number of her offspring survive to reproduce.

The length of time required for eggs to hatch influenced size and survivorship of larvae. Eggs that required a longer time to hatch resulted in larvae experiencing lower survivorship after 40-day growth. After 80-day growth, the longer that eggs took to hatch, the larger the resulting larvae. Although I do not know what factor(s) contribute to differences in days required for hatch, this variable significantly influenced size and survivorship of larvae early in larval development (40 and 80 days growth).

The coupled sediment experiments (larval size/survivorship and sediment selection by larvae) indicated that Saginaw Bay Sediment was the sediment type that supported larvae that attained the largest size and had the highest survivorship. Additionally, larvae selected Saginaw Bay when

given a choice among different sediment types. A standard sediment (STND-3) consisting of potting soil, silica sand, and sculptor's clay that mimicked the sediment particle size distribution and organic content of Saginaw Bay sediment was developed. A combination of particle size distribution and organic content of Saginaw Bay sediment presumably results in the best cohesive sediment for burrow production.

The experiment that compared larval size and survivorship among three standard sediments and Saginaw Bay revealed that STND-3 sediment produced larvae with the highest survivorship. I therefore recommend STND-3 sediment as a suitable laboratory sediment for rearing Hexagenia. The low contaminant burden of this sediment also indicates that this sediment type is appropriate for use in bioassay studies.

Results of the study that compared two different food types indicated that there was no difference in size and survivorship of larvae fed the two food types; larvae grew equally well when fed either Tetramin^R, yeast and alfalfa or Tetramin^R and yeast only. Therefore, either food mixtures could be used for successful rearing of Hexagenia larvae.

The mass rearing experiment was successful in that large numbers of mayflies were reared from first instar larvae to adults or late instar larvae within three months. To ensure high survivorship of half-grown larvae, I recommend rearing larvae for no longer than 10 weeks to avoid larvae that are either in the last stage of development (i.e., black wing pad stage) or emerged as subimagos.

I recommend using photographs as a means to estimate larval size, when measuring actual larvae is not possible. Photographs provide a permanent, accurate record of the number and size of larvae.

Because body size is so variable in Hexagenia, and because all life stages of this mayfly are both identifiable and measurable, this insect is an excellent model organism for life history studies. Other studies are required to further elucidate many of the relationships that I have examined in this thesis. The protocols that I have developed for laboratory rearing this mayfly, if widely used, should greatly facilitate comparison of these studies.

Future Research Needs

I believe the following laboratory and field studies are required to better elucidate many of the relationships that I examined in this thesis.

A laboratory experiment designed to examine the environmental cues (i.e., temperature, photoperiod) responsible for stimulating emergence may explain why the size of female subimagos decreases over the emergence period.

It would be helpful to know if eggs overwinter in field populations to determine if this variable contributes to size variation of larvae. To test for overwintering of eggs, sampling field sediments in spring would be required. The

presence of viable Hexagenia eggs or first instar larvae would indicate that this phenomenon occurs.

In the artificial insemination experiment, when egg hatching occurred in all treatments (i.e., eggs fertilized with H. limbata sperm, H. rigida sperm, or not fertilized) I assumed that the females in that treatment had previously been mated. However, since I collected subimagos from the field for this experiment, the implication is that either subimago females are mating in the field, or imagoes are mating in the laboratory. Both of these events have not been previously documented for Hexagenia. Collecting subimagos at the same time that swarming is occurring, and checking for either presence of sperm on eggs or monitoring hatch would reveal if the subimagos were mating in the field.

It is evident from my research that maternal size does not necessarily dictate a female's allocation of her resources to egg size and first instar size (indicated by results of maternal size and egg size and first instar size study), and yet these two variables (egg size and first instar size) do vary among females. A detailed study of maternal investment should include measurements of clutch size, egg size, and first instar size for each female. This approach would allow an individual's investment into reproduction to be monitored, and the allocation of resources into egg number and size to be determined.

A laboratory experiment designed to examine the influence of maternal size and emergence date on size and survivorship

of offspring in which the larvae were reared to adult would be useful. Results of this proposed study would reveal whether observed differences in larval size/survivorship that occur early in development (40, 80 day growth) were indicative of size of emerging subimagoes.

A laboratory experiment designed to examine the influence of container size on offspring growth would allow results of experiments conducted in small containers to be compared to more natural conditions. This is especially important since many laboratory experiments are conducted using small sized containers, which is often a function of limited laboratory space and large numbers of replicates required per treatment.

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